Cloning and Characterization of the cDNA Coding for a Polymyositis-Scleroderma Overlap Syndrome-related Nucleolar 100-kD Protein

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Summary

About 50% of patients with the polymyositis-scleroderma overlap syndrome are reported to have autoantibodies to a nucleolar particle termed PM/Scl. The particle consists of several polypeptides of which two proteins of 75 and 100 kD have been identified as the major antigenic components. Here we report on the cDNA cloning and partial epitope mapping of the 100-kD autoantigen from human placenta and HeLa λgt11 libraries. The deduced amino acid sequence encodes a protein of 885 amino acid residues with a molecular mass of 100.8 kD. Rabbit antibodies raised against a recombinant protein fragment reacted in immunofluorescence and immunoblotting in the same manner as human autoantibodies directed against the nucleolar 100-kD protein. Sequence analysis shows close homology to a consensus sequence of 12 amino acids from serine/threonine kinases, suggesting a possible function for this autoantigen. A major antigenic region is found to be located within the NH2-terminal third of the polypeptide.

A variety of antinucleolar autoantibodies have been reported to occur in sera from patients suffering from systemic sclerosis (scleroderma). The antigens reacting with these patient sera are RNA polymerase I, DNA topoisomerase I, fibrillarin, and a so far unidentified nucleolar particle that is precipitated by a high percentage of sera from patients with the polymyositis-scleroderma (PM/Scl)1 overlap syndrome. Antibodies to the PM/Scl antigen are found predominantly in sera from patients with clinical features of scleroderma (3%) and polymyositis (8%). A significantly higher incidence (50%) of PM/Scl autoantibodies was observed for patients with the PM/Scl overlap syndrome (1). Indirect immunofluorescence studies have shown that the PM/Scl autoantibodies give strong staining of the cell nucleolus with some weak staining of the nucleoplasm (2–4). Further studies indicated that the PM/Scl antigen is localized in the granular component of the nucleolus (3, 5).

By immunoprecipitation it could be shown that the PM/Scl particle consists of ~11–16 polypeptides with apparent molecular masses ranging from 20 to 100 kD (3, 4, 6, 7). Although no RNA was found to be precipitated with the particle, its location in the granular component of the nucleolus suggests its involvement in ribosome maturation (3, 8). In immunoblots most of the PM/Scl sera were found to react with polypeptides of 75 and 100 kD. Since no crossreactivity for the two immunoreactive peptides could be observed with affinity-purified antibodies, it was assumed that they represent two distinct protein entities (7).

Recently, Alderuccio et al. (8) succeeded in cloning and sequencing of the human cDNA encoding the 75-kD autoantigen of the PM-Scl complex. In this study we report the application of affinity-purified antibodies from the PM/Scl 100-kD protein in the cloning and sequencing of the human cDNA encoding the 100-kD autoantigen. We have cloned the full-length cDNA of 2,797 bp coding for a polypeptide of 885 amino acids with a deduced molecular mass of 100.8 kD.

Materials and Methods

Patients and Sera. 15 sera from patients suffering from PM/Scl overlap syndrome were kindly provided by Dr. E. Genth (Rheumaklinik, Aachen, Germany). Two sera from patients with other autoantibody profiles and a normal human serum were used as controls.

Preparation of Nucleolar Extracts. Nuclei of 1.5 × 109 HeLa S3 cells were prepared as described previously (9). All buffers contained 1 mM PMSF and all steps were done at 4°C. Pelleted and washed nuclei were sonicated in 15 ml buffer I (250 mM sucrose, 10 mM Tris/Cl, pH 7.4, 1 mM MgCl2) with five 5-s pulses at 45 W using a sonifier (Bronson Sonic Power Co., Danbury, CT). The suspension was centrifuged through a sucrose cushion (880 mM sucrose, 10 mM Tris/Cl, pH 7.4) at 1,000 g and 4°C for 30 min. The supernatant was discarded, and the nucleoli were resuspended in 4 ml buffer II (880 mM sucrose, 10 mM Tris/Cl, pH 7.4, 1 mM MgCl2) and sonicated as described above. Disrupted nucleoli were pelleted at 4,000 g and 4°C for 20 min and resuspended in 2 ml Laemmli buffer.

1 Abbreviation used in this paper: PM/Scl, polymyositis-scleroderma.
Screening of cDNA Libraries with Antibody Probes. Affinity-purified anti-100-kD antibodies from serum A3 were used to screen a total of $2.5 \times 10^6$ recombinant phages from HeLa cDNA libraries constructed with $\lambda$ gt11 (10) (Genoñit, Heidelberg, Germany). Two nitrocellulose sheets were used on each plate. The first was probed with undiluted affinity-purified antibodies and the second with patient's serum A3 (1:800 in TBS). Only plaques that were positive on both sheets were further purified.

Screening of cDNA Libraries with DNA Probes. After EcoRI digestion and separation by agarose gel electrophoresis, cDNA fragments were purified using the GeneClean kit (Dianova, Hamburg, Germany). Fragments were labeled with Digoxigenin (Boehringer, Mannheim, Germany) and used as hybridization probes (following manufacturer's instructions) on a total of $2.5 \times 10^6$ recombinant phages from human placenta cDNA libraries in $\lambda$ gt11 (Genoñit).

DNA Sequencing. DNA fragments were subcloned into BlueScript SK or KS vector (Stratagene, Heidelberg, Germany), M13mp18, and M13mp19. DNA sequencing was carried out according to the dideoxy technique of Sanger et al. (11) using synthetic oligonucleotide primers. The nucleotide sequences were analyzed with PC/Genetm software (IntelliGenetics, Inc., Mountain View, CA).

Polymerase Chain Reaction. DNA fragments for partial epitope mapping were generated by PCR (12). Primers used carried either a synthetic BamHI or BglII site and a three-nucleotide clamp at their 5' ends. Asymmetric PCR was performed according to McCabe (13) using a molar ratio of primers of 75:1.

Analysis of the mRNA 5' End by PCR. The 5' sequence was analyzed by reverse transcription of HeLa S3 mRNA with a gene-specific primer followed by tailing and PCR as described previously (14). The resulting DNA fragment was amplified again by asymmetric PCR (13). The single-stranded DNA was sequenced by the chain termination method using a gene-specific antisense primer ranging from nucleotide 279 to 263.

Expression of Recombinant Proteins. The DNA fragment from clone A16/2 was subcloned from pBluescript into pUR291 and the corresponding fusion protein was expressed as described (15). For epitope mapping PCR-generated fragments were subcloned into the BglII site of pDS56-6His vector (16). Expression of the peptide fragments was induced with 2 mM isopropyl-β-D-thiogalactoside (IPTG) and purification of the peptides was performed on Ni²⁺ chelate columns as described (16).

Immunoblotting and Affinity Purification of Antibodies. Protein samples were subjected to electrophoresis on SDS-polyacrylamide gels (12.5, 15, or 17.5%, respectively) (17) and electroblotted onto nitrocellulose (18). The blots were blocked with 40% horse serum for 90 min and probed with the appropriate serum dilution. Bound antibodies were visualized with a second antibody (goat anti-human IgG(H+L) or goat anti-rabbit IgG(H+L)) (Dianova) coupled to alkaline phosphatase. Affinity purification of bound antibodies from nitrocellulose sheets was performed by the pH shock method as described by Smith and Fisher (19) followed by concentration with Centricon-30 microconcentrators (Amicon Corp., Danvers, MA).

Indirect Immunofluorescence. Cytoimmunofluorescence was performed on Hep2 cells grown on cover slips and fixed by methanol and acetone as described (20). They were then incubated either with anti-PM/Scl serum, affinity-purified anti-PM/Scl antibodies, or affinity-purified rabbit antibodies in PBS, pH 7.4, in appropriate dilutions, and subsequently with FITC-conjugated goat anti-human IgG (Dianova) or goat anti-rabbit IgG in the same buffer. Immunofluorescence was observed (Axioskop, Plan-Neofluar 40x, equipped with epifluorescence optics; Zeiss, Oberkochen, Germany) and photographed with Ilford (Mobberly Cheshire, England) HP5 film.

Immunization of Rabbits with a Purified Protein Fragment. New Zealand white rabbits were bled before immunization and then immunized subcutaneous in the neck with FPLC-purified peptide fragment A (150 µg) in ABM-S (Linaris, Bettingen, Germany) as adjuvant. Second and third immunizations were given after 15 and 27 d with 150 µg antigen each. Rabbits were bled after 40 d, and immunoreactivity was tested in indirect immunofluorescence and immunoblotting.

Results

Immunoblotting Analysis of PM/Scl Autoantibodies. The PM/Scl autoantibodies used in this study revealed immunoreactivity with two major nucleolar antigens with molecular masses of 75 and 100 kD, as determined by immunoblot analysis on nucleolar extracts (Fig. 1, lanes a and b). Occasionally, an additional reactivity was observed with an 80-kD nucleolar antigen (data not shown). These data are in good agreement with previously published results (3, 8).

Isolation and Characterization of a Partial cDNA Clone. Antibodies were affinity purified from the nucleolar 100-kD protein and used to screen $2.5 \times 10^6$ recombinants from a HeLa $\lambda$ gt11 expression library. After plaque purification four clones were obtained that on further investigation were shown to be identical. The respective β-galactosidase fusion proteins had a molecular mass of 135 kD, as determined in SDS-PAGE (data not shown). Therefore, the cDNAs encoded a peptide fragment of ~20 kD. All four fusion proteins, but not β-galactosidase alone, reacted with anti-PM/Scl serum A3 in immunoblot analysis (data not shown). Expression in

Figure 1. Immunoreactivities of selected PM/Scl patient sera with the nucleolar 100-kD protein. Immunoblots of HeLa nucleolar extracts separated on 12.5% SDS-polyacrylamide gels were probed. (Lane a) PM/Scl patient serum A2; (lane b) PM/Scl patient serum A3; (lanes c and d) a normal human and a primary biliary cirrhosis (PBC) patient serum as controls. The 100- and 75-kD nucleolar proteins of the PM/Scl complex are indicated.
Figure 2. Immunoblot analysis of a HeLa nucleolar extract separated on a 12.5% SDS-polyacrylamide gel. (Lane a) Antibodies of patient serum A3 affinity purified from β-galactosidase; (lane b) patient serum A3. The fusion protein from clone A16/2 was used to affinity purify antibodies from patient serum A3 (lane c) or normal human serum (lane d). The position of the 100-kD nucleolar protein is indicated.

pUR291 and further immunoblotting analysis of the respective β-galactosidase fusion protein confirmed an open reading frame as determined by sequence analysis (nucleotide position 235–745). Affinity-purified antibodies from the fusion protein were tested by immunoblotting and indirect immunofluorescence. In immunoblots only the nucleolar 100-kD protein is recognized by the affinity-purified antibodies (Fig. 2, lane c). Indirect immunofluorescence studies with these antibodies showed identical nucleolar staining on Hep2 cells as affinity-purified 100-kD antibodies (Fig. 3, a and b). These results confirm that our cDNA clone codes for a fragment of the 100-kD PM/Scl antigen. All six PM/Scl sera tested reacted with this fusion protein in immunoblots. No reaction was observed with a human control serum (data not shown), suggesting that we have cloned a major antigenic region of the 100-kD PM/Scl antigen.

Isolation and Characterization of a Full-Length cDNA Clone. The cDNA fragment of clone A16/2 was labeled with digoxigenin and used as a hybridization probe to isolate a full-length cDNA from a human placenta cDNA library in λgt11. Screening of 2.5 × 10^6 recombinants yielded three clones with cDNA inserts of the expected sizes. The corresponding cDNAs were subcloned into M13mp18 and M13mp19 and sequenced. The sequencing strategy is shown in Fig. 4 a. One clone (H2/8) was obtained that presumably encoded the full-length cDNA of the 100-kD PM/Scl antigen ranging from nucleotide -1 to 2797. The nucleotide sequence and the deduced amino acid sequence are shown in Fig. 4 b. A long open reading frame was found, ranging from position 1 to 2655, that encodes 885 amino acids with a predicted molecular mass of 100.8 kD, which corresponds well to the observed molecular mass of 100 kD of the nucleolar protein. The termination codon TAG is at position 2656. A potential polyadenylation signal (ATTAAA) is located at position 2737, followed by the poly(A) tail starting at position 2753.

Determination of the 5′ End of the mRNA. To determine the 5′ end of the transcription product, mRNA from HeLa S3 cells was isolated and cDNA was synthesized, followed by tailing, PCR, and sequencing of the resulting single-stranded DNA fragment, as described in Materials and Methods. The obtained sequence exactly matched the sequence of clone H2/8 and showed 36 additional nucleotides from position -37 to -1 (Fig. 4 b). No termination signal was observed preceding the putative start codon ATG at position +1. However, the sequence surrounding the start codon ATG at position +1 matches Kozak sequence elements (21). These results, together with the fact that no longer cDNA could be isolated from the cDNA libraries, indicate that we have cloned the full-length cDNA of the human 100-kD PM/Scl autoantigen.

Figure 3. Indirect immunofluorescence on Hep2 cells with affinity-purified anti-PM/Scl 100-kD antibodies from patient serum A3. Antibodies were eluted from the nucleolar 100-kD protein bound to nitrocellulose (a) and from the fusion protein of clone A16/2 (b). Bound antibodies were visualized with a FITC-coupled goat anti-human IgG antibody, and immunofluorescence was observed by epifluorescence optics (×750).
Figure 4. cDNA sequence of clone H2/8 and deduced amino acid sequence of the encoded nucleolar 100-kD PM/Scl protein. (a) Sequencing strategy of clone H2/8. Sequencing was carried out by the Sanger dideoxy technique using synthetic oligonucleotide primers (see Materials and Methods). (b) cDNA sequence of the 100-kD PM/Scl clone H2/8. The deduced amino acid sequence is displayed below the DNA sequence. The start and termination codons and the potential polyadenylation signal (ATTAAA) are underlined. Double-underlined sequences show the additional eDNA sequence obtained by reverse transcription followed by tailing, PCR, and direct sequencing of the resulting single-stranded PCR product (see Materials and Methods). The sequence data are available from the EMBL Data bank under accession number X66113.
Table 1. Amino Acid Sequence Comparison of the 100-kD PM/Scl Protein and Serine/Threonine Kinase Sequences (22-29)

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<th>Amino acid residue</th>
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<th>Sequence</th>
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<td>E T R L L H A K N I I R P Q L K F</td>
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<td>457-472</td>
<td>C G L Q F L H G K G I I Y R D L K L</td>
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<tr>
<td>Potential consensus sequence</td>
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Relevant segments of the serine/threonine kinase sequences are compared with the corresponding segment of the nucleolar 100-kD PM/Scl protein. The amino acid positions are shown together with the respective sequences. The regions representing the potential consensus sequence are boxed. The potential consensus sequence itself is displayed below the aligned sequences.

**Computer Search for Sequence Similarities of the PM/Scl 100 Antigen.** The deduced amino acid sequence of the 100-kD protein was used to search the EMBL data bank for homologous sequences. No close similarity was found with any reported sequence, implying that we have cloned a new and unique cDNA. However, a stretch of 9 of 12 consecutive amino acids (amino acid residues 175–186) could be found to show identity with the consensus sequence of a series of proteins, all belonging to the serine/threonine kinase family (22-29). All together, 28 serine/threonine kinases were found to contain this or a related subsequence. Table 1 shows a computer-based alignment of some representatives of this group of proteins together with the corresponding amino acid sequence of the 100-kD PM/Scl autoantigen. Further analysis of the cDNA sequence by the PC/Gene™ program showed 16 potential phosphorylation sites for serine/threonine kinases on our deduced amino acid sequence, in contrast to only two potential phosphorylation sites for tyrosine kinases.

**Expression of Defined cDNA Fragments and Partial Epitope Mapping.** For partial epitope mapping, DNA fragments were prepared by PCR using synthetic oligonucleotide primers as described in Materials and Methods (Fig. 5 a). The resulting cDNA fragments were subcloned into the BgIII site of pDS56-6His vector. Expression and purification of the corresponding peptide fragments were performed as described in Materials and Methods. The reactivities of the PM/Scl sera with the various peptide fragments were determined by immunoblots (Fig. 5 b) and are summarized in Table 2. All sera recognizing the 100-kD nucleolar protein react with the peptide fragments A, B, and C, indicating that at least one major antigenic determinant is localized between amino acid residues 153 and 324. Peptide fragments E and F are recognized by 4 of 15 sera. This indicates that at least one minor antigenic determinant is localized between amino acid residues 492 and 703. Peptide fragment D is not recognized by any of the sera. The relative positions of the fragments and the antigenic determinants are shown in Fig. 6.

**Rabbit Antibody to a Recombinant Peptide.** The peptide fragment A (Figs. 5 and 6, lane a) was used to raise antibodies in rabbits as described in Materials and Methods. The resulting antibodies were tested in indirect immunofluorescence and in immunoblots. Antibodies affinity purified from peptide fragment A were used for further studies.

In indirect immunofluorescence on Hep2 cells, nucleolar staining could be observed (Fig. 7 a). In immunoblots the affinity-purified rabbit antibodies reacted with the 100-kD nucleolar protein (data not shown). These findings give further support to the authenticity of our cloned cDNA to code for the 100-kD PM/Scl autoantigen.

Figure 5. Determination of antigenic regions by immunoblotting of the expressed peptide fragments of the 100-kD PM/Scl protein. (a) Exact amino acid positions of the peptide fragments tested. (b) Representative immunoblot of the peptide fragments A-F using patient serum A14.
Table 2. Summary of the Reactivities in Immunoblot Analysis of the PM/Scl Patient and Control Sera with the Expressed Peptide Fragments of the Nucleolar 100-kD PM/Scl Protein

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<th>Serum*</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
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* Sera A1–A14, L, H, and K are PM/Scl patient sera; immunoreactivities: ++, strong positive reaction; +, positive reaction; (+), weak reaction; -, no reaction.
† 100 kD is the nucleolar 100-kD PM/Scl protein.
§ Control sera: NHS is a normal human serum (Blutbank, Heidelberg, Germany); PBC I and II are sera from patients with primary biliary cirrhosis.

Discussion

The aim of the work reported here was the cloning and characterization of the cDNA coding for the nucleolar 100-kD protein, which was found to be the major antigen recognized by PM/Scl autoantisera (4).

We have cloned the full-length cDNA of this protein with 2797 bp coding for 885 amino acid residues with a deduced molecular mass of 100.8 kD. A comparison with the recently published cDNA sequence of the nucleolar 75-kD PM/Scl protein (8) showed no homology to the cDNA sequence of the 100-kD PM/Scl protein. This result confirms our previous observation that affinity-purified 100 kD autoantibodies do not crossreact with the 75-kD nucleolar protein and vice versa (7).

The sequence identity in 9 of 12 consecutive amino acids with several proteins belonging to the serine/threonine kinase family indicates a possible function for the nucleolar 100-kD protein (Table 1). As protein kinases are known to be involved in many regulatory processes, our results are in agreement with the suggestion made by several investigators that the minor antigenic determinant(s) as determined by immunoblot analysis.
that the PM/Scl particle is involved in ribosome maturation due to its localization in the granular component of the nucleolus (3, 8), its presence in the nucleoplasm, its absence from the cytoplasm, and the apparent migration of nucleolar staining to the nucleoplasm after actinomycin D treatment (4). Previously described proteins found or thought to be involved in ribosome biogenesis, such as the 40-kD protein ribocharin (30), a 100-kD protein known as nucleolin (31), and a 94-kD protein localized in the dense fibrillar component of the nucleolus (32), seem to be distinct from the PM/Scl proteins.

One of our main interests, however, was the localization of the antigenic regions on the 100-kD nucleolar protein. Experiments using 15 PM/Scl sera uncovered at least one antigenic domain between amino acid residues 153 and 324, represented by peptide fragment C. Another minor antigenic domain appears to occur between amino acid residues 492 and 703, as 4 of 15 PM/Scl sera immunoreacted with the peptide fragments E and F. These results are in reasonable agreement with a computer-based analysis of hydrophilic regions on the deduced amino acid sequence of the 100-kD protein. In this context we would like to point out that the experimental setup used here did not allow us to uncover nonlinear epitopes.

Brendel et al. (33) observed that antigens associated with systemic autoimmune diseases possess one of the longest charge runs found in proteins sequenced to date (33). In this context it is interesting to note that the antigen described here possesses two relatively long stretches (amino acid residues 218–232 and 795–814) of a high density of charged amino acids.

In summary, we describe the cloning of a 2,797-bp cDNA encoding the 100-kD polypeptide of the PM/Scl particle. At least one major antigenic determinant is located in the NH₂-terminal third of the protein. Rabbit antibodies raised against a recombinant protein fragment show the same immunological characteristics as human PM/Scl autoantibodies. The presence of a sequence showing a high homology with a consensus sequence present in several serine/threonine kinases suggests a possible function for the 100-kD protein.

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