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The C-terminal RG Dipeptide Repeats of the Spliceosomal Sm Proteins D1 and D3 Contain Symmetrical Dimethylarginines, Which Form a Major B-cell Epitope for Anti-Sm Autoantibodies*

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The Sm proteins B/B′, D1, D2, D3, E, F, and G are components of the small nuclear ribonucleoproteins U1, U2, U4/U6, and U5 that are essential for the splicing of pre-mRNAs in eukaryotes. D1 and D3 are among the most common antigens recognized by anti-Sm autoantibodies, an autoantibody population found exclusively in patients afflicted with systemic lupus erythematosus. Here we demonstrate by protein sequencing and mass spectrometry that all arginines in the C-terminal arginine-glycine (RG) dipeptide repeats of the human Sm proteins D1 and D3, isolated from HeLa small nuclear ribonucleoproteins, contain symmetrical dimethylarginines (sDMAs), a posttranslational modification thus far only identified in the myelin basic protein. The further finding that human D1 individually overexpressed in baculovirus-infected insect cells contains asymmetrical dimethylarginines suggests that the symmetrical dimethylation of the RG repeats in D1 and D3 is dependent on the assembly status of D1 and D3. In antibody binding studies, 10 of 11 anti-Sm patient sera tested, as well as the monoclonal antibody Y12, reacted with a chemically synthesized C-terminal peptide of D1 containing sDMA, but not with peptides containing asymmetrically modified or nonmodified arginines. These results thus demonstrate that the sDMA-modified C terminus of D1 forms a major linear epitope for anti-Sm autoantibodies and Y12 and further suggest that posttranslational modifications of Sm proteins play a role in the etiology of systemic lupus erythematosus.

The small nuclear ribonucleoproteins (snRNPs)[1] U1, U2, U5, and U4/U6 are the major functional constituents of the spliceosome, a nuclear multicomponent complex that catalyzes the splicing of nuclear pre-mRNA in eukaryotes (1). The UsnRNPs consist of one (in the case of U1, U2, and U5) or two (in the case of U4/U6) UsnRNAs and at least 50 distinct proteins (2). These proteins can be divided into two groups: the Sm proteins B/B′, D1, D2, D3, E, F, and G, found in each of the UsnRNPs, and the specific proteins that are associated with only one of the UsnRNP particles.

The Sm proteins play a key role in the biogenesis of the UsnRNPs. In the cytoplasm, the Sm proteins form RNA-free hetero-oligomers E·F·G, D1·D2, and B/B′·D3 (3–6). After the nuclear export of the RNA polymerase II transcripts U1, U2, U4, and U5, the Sm protein complexes assemble on the Sm site, a conserved sequence that these UsnRNAs have in common (7). Interestingly, single Sm proteins do not bind to the UsnRNA. Rather, the D1·D2 and E·F·G complexes first bind to the Sm site, thus forming a so-called subcore snRNP intermediate. Subsequent association of the B/B′·D3 complex completes the formation of the core UsnRNP. The core UsnRNP is required for the hypermethylation of the m^7G cap of the U1, U2, U4, and U5 snRNAs to m_3G caps (8, 9) and also constitutes part of the bipartite nuclear localization signal necessary for the import of the UsnRNPs into the nucleus (10, 11). Moreover, there is evidence that the Sm proteins may stabilize the association of specific snRNP proteins (12).

Sm proteins are not only of functional interest but also of clinical relevance. Patients suffering from the autoimmune disease systemic lupus erythematosus (SLE) spontaneously produce autoantibodies against a multitude of cellular components (13–15). Antibodies against Sm proteins (anti-Sm autoantibodies) are SLE-specific, in contrast to most other SLE-associated autoantibody populations. Therefore, the elucidation of the antigenic determinants recognized on the Sm proteins is of significant diagnostic and immunopathological relevance.

In immunoblots, anti-Sm autoantibodies react predominantly with the Sm proteins B/B′, D1, D3, and, to a lesser extent, D2 (16, 17). The E, F, and G proteins are also frequently recognized under native conditions, such as in immunoprecipitation assays (18). The parallel recognition of several Sm proteins by anti-Sm patient sera is not only due to distinct antibody populations but also to cross-reactive epitopes on the Sm proteins (16, 19), thus indicating that the Sm proteins share structural homology. Indeed, all Sm proteins are evolutionarily conserved in an approximately 70–90-amino-acid-long region designated the Sm domain, which contains the Sm1 and Sm2 motifs (3, 20, 21). The recent elucidation of the crystal structures of the B·D3 and D1·D2 complexes demonstrated that the

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[1] The abbreviations used are: snRNP, small nuclear ribonucleoprotein; SLE, systemic lupus erythematosus; sDMA, symmetrical dimethylarginine; aDMA, asymmetrical dimethylarginine; MMA, monomethylarginine; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; LIA, line immunoassay; MBP, myelin basic protein; Fmoc, *N*-(9-fluorenyl)methoxycarbonyl; PTH, phenylthiohydantoin; HPLC, high pressure liquid chromatography; ACTH, adrenocorticotropic hormone; UsnRNA, uridyl-rich small nuclear RNA; UsnRNP, uridyl-rich small nuclear ribonucleoprotein.

fold of the Sm domain is also highly conserved (22). Epitope mapping studies with the Sm proteins B/B′ and D1 have demonstrated that the Sm domains of these proteins indeed form discontinuous B-cell epitopes (23, 24). However, some putative linear epitopes have also been mapped on the Sm proteins D1 and B/B′. These epitopes are predominantly located in the C-terminal extensions of the D1 and B/B′ proteins and not in the Sm domain (25). The C terminus of D1, for example, which contains a stretch of nine RG dipeptides (26), was reported to form an epitope for anti-Sm autoantibodies (23, 27–30). However, in contrast to the native D1 protein, the C-terminal epitope of D1 often reacted with autoantibodies from patients with other diagnoses than SLE (30). Therefore, the disease-specific recognition of the D1 C terminus by anti-Sm autoantibodies seems not only to be sequence-dependent but could additionally depend on other factors such as proper folding and/or posttranslational modifications.

C-terminal RG dipeptide repeats are found in D1 and D3 orthologs of most organisms except yeast, suggesting that the RG stretches of D1 and D3 play an important functional role in higher eukaryotes. RG-rich regions are also found in several other nuclear proteins that are involved in RNA processing (*e.g.* heterogeneous nuclear ribonucleoprotein A1, fibrillarin, nucleolin, and the yeast-RNA binding protein Np/3p (for a review, see Ref. 31)). However, in contrast to D1 and D3, they contain RGG motifs. Interestingly, the glycine/arginine-rich motifs of these proteins commonly contain asymmetrical dimethylarginines (aDMAs), a posttranslational modification formed by type I protein arginine *N*-methyltransferases (31). We surmised that methylation of arginines in D1 could not only explain why synthetic RG-rich D1 peptides often react unspecifically (30) but also why human D1 protein reacts only weakly and sometimes unspecifically with anti-Sm autoantibodies when overexpressed in *E. coli* (32–34). We therefore analyzed the Sm proteins D1 and D3 for posttranslational modifications and investigated the influence of such modifications on their antigenicity. Using protein sequencing and mass spectrometry, we could demonstrate that the human Sm proteins D1 and D3 are dimethylated *in vivo* in all nine and four positions, respectively, of their C-terminal RG-stretches. In contrast to other nuclear proteins, D1 and D3 contain symmetrical dimethylarginines (sDMAs), a posttranslational modification thus far only identified in the myelin basic protein (MBP). In epitope mapping studies, a synthetic C-terminal peptide of D1 that contained sDMA reacted with 10 of 11 anti-Sm patient sera and the monoclonal antibody Y12, while homologous peptides with aDMA or nonmodified arginines were not recognized. Our data thus demonstrate that the recognition of D1 by anti-Sm autoantibodies and Y12 is strongly dependent on the symmetrical dimethylation of the C terminus of D1.

EXPERIMENTAL PROCEDURES

Patient Sera—Sm-positive patient sera ($n = 11$) were obtained from the Department of Rheumatology, Ghent University Hospital (Ghent, Belgium). Selection of Sm-positive sera was based on the use of the INNO-LIA™ ANA (Innogenetics N.V., Ghent, Belgium), a line immunoassay (LIA) containing natural SmD derived from purified HeLa UsnRNPs. Control sera tested negative for anti-nuclear factor in indirect immunofluorescence on Hep2 cells and showed no reactivity on the INNO-LIA™ ANA.

Isolation of Sm Proteins and Protein Sequencing—Purification of HeLa UsnRNPs and subsequent separation of Sm proteins by SDS-polyacrylamide gel electrophoresis have been described earlier (16). Coomassie Blue-stained bands of the respective Sm proteins were excised and directly submitted to proteolytic digestion according to Rosenfeld *et al.* (35). Briefly, the excised gel slices were washed twice with 2 ml of water for 1 h and then shaken strongly in an aqueous solution (pH 6.4) of 40% acetone, 10% triethylamine, and 5% acetic acid for 2 h. After washing three times for 1 h with 2 ml of water, the gel slices were dried and proteolytically digested with 0.5–2.0 μg of endoproteinase Lys-C (Roche Molecular Biochemicals) in 200–500 μl of digestion buffer (100 mM Tris-HCl, pH 8.0, 10% acetonitrile, 1 mM K_3EDTA). The peptide mixture was separated by HPLC on a C4 column (Vydac, Hesperia, CA) using a 10–70% gradient of solvent B (70% acetonitrile, 0.1% trifluoroacetic acid). Manually recovered peptide fractions were then sequenced by Edman degradation using a Procise 492 sequencer equipped with an on-line 140C phenylthiohydantoin analyzer (Perkin-Elmer). To distinguish methylated arginines from nonmethylated arginines, we first applied a combination of either monomethylarginine (MMA) and sDMA or MMA and aDMA to a biobrene-coated, trifluoroacetic acid-treated glass fiber filter (Perkin-Elmer) and performed an Edman degradation cycle for each combination. Next, the human C-terminal D1 peptide, the C-terminal D3 peptide, or the baculo-derived C-terminal peptide was applied on the same filter and sequenced to completion. All additional standards were purchased from Sigma.

Overexpression and Purification of D1 in Escherichia coli and Baculovirus-infected Insect Cells—A cDNA containing the sequence of human D1 (26) was inserted as a *Bam*HI/*Xba*I fragment into the expression vector pIGFH111 (Innogenetics), thus introducing the first 25 amino acids of mouse tumor necrosis factor and six histidines to the amino terminus of D1. After transfection into the *E. coli* strain SG4044 (pc1857) and temperature induction, a strong protein band in the expected range of 18 kDa could be detected in the soluble fraction. This protein was verified to be recombinant D1 by immunoblotting with an anti-tumor necrosis factor antibody (Innogenetics). After lysing the cells by French pressing in lysis buffer (10 mM Tris-HCl, pH 6.8, 100 mM KCl, 25 mM ϵ-aminocaproic acid, 1 mM dithiothreitol, 2 mM phenylmethylsulfonyl fluoride) and centrifugation at 4 °C (20 min at 27,000 × *g*), guanidinium HCl was added to the supernatant to a final concentration of 4.5 M, and recombinant protein was purified by affinity chromatography on a nickel column (nickel-Sepharose; Amersham Pharmacia Biotech).

For overexpression of D1 in baculovirus-infected insect cells, a 520-base pair *Dra*I–*Sba*I fragment of pIGFH111hSmD1 (see above) was inserted into a *Bam*HI–*Xba*I-digested baculovirus transfer plasmid pVL1393 (Pharmingen, San Diego, CA) in which the fusion gene is under transcriptional control of the strong baculovirus polyhedrin promotor. After transfection of the vector (as described by the manufacturer), a D1 protein was expressed, as evidenced by immunoblotting (see above). After lysis, baculo-derived D1 was purified on a nickel column as described above.

Mass Spectrometry—Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry of the C-terminal D1 peptide was performed on a Voyager-DE STR (PerSeptive Biosystems, Framingham, MA) mass spectrometer equipped with delayed extraction and operated at an accelerating voltage of 20 kV and 150-ns delay. Desorption and ionization of the sample was carried out with a nitrogen laser (337 nm). Samples were applied on a stainless steel sample plate using the thin layer method (36) and measured in reflectron mode. MALDI-TOF-obtained spectra were externally calibrated using a standard peptide mixture of angiotensin I 1296.6853 $[M + H]^+$, ACTH-(1–17) 2093.0867 $[M + H]^+$, ACTH-(18–39) 2465.1989 $[M + H]^+$, and ACTH-(7–38) 3657.9294 $[M + H]^+$. The observed mass peaks were annotated manually. Electrospray mass spectrometry of recombinant D1 was performed in a Bio-Q-quadrupole mass spectrometer equipped with an electrospray ion source (Micromass, Wytenshawe, United Kingdom). For analysis, 20 pmol of protein or peptide were applied in 10 μl of 50% acetonitrile, 1% acetic acid. Calibration was performed with 50 pmol of horse heart myoglobin.

Peptide Synthesis and LIA—Peptides were synthesized on Tentagel S resin (Rapp Polymere GmbH, Germany) using a Rainin Symphony Multiplex synthesizer (Protein Technologies, Tucson, AZ) with standard Fmoc chemistry. Standard double couplings were performed by using a 4-fold excess of Fmoc-protected amino acids activated *in situ* with equimolar amounts of *N*-hydroxybenzotriazole and 2-(1H-benzotriazole-1-yl)-1,1,3,3,-tetramethyluronium tetrafluoroborate twice for 20 min each time. Fmoc-protected asymmetrical and symmetrical dimethylarginine (Bachem, Switzerland) were incorporated similarly. After completion of the peptide synthesis, the peptide was cleaved from the resin by incubation for 2.5 h with a mixture of 90% trifluoroacetic acid, 5% thioanisole, 3% ethanedithiol, and 2% anisole. The peptide was precipitated from the mixture using *t*-butyl methyl ether. After centrifugation, the pellet was washed three times with *t*-butyl methyl ether and dried overnight in a vacuum. The purity of the crude peptide was checked by reversed-phase HPLC.

For LIA, purified, gel-eluted HeLa proteins, from which SDS and Coomassie were removed by ion pair extraction (16) or purified recom-

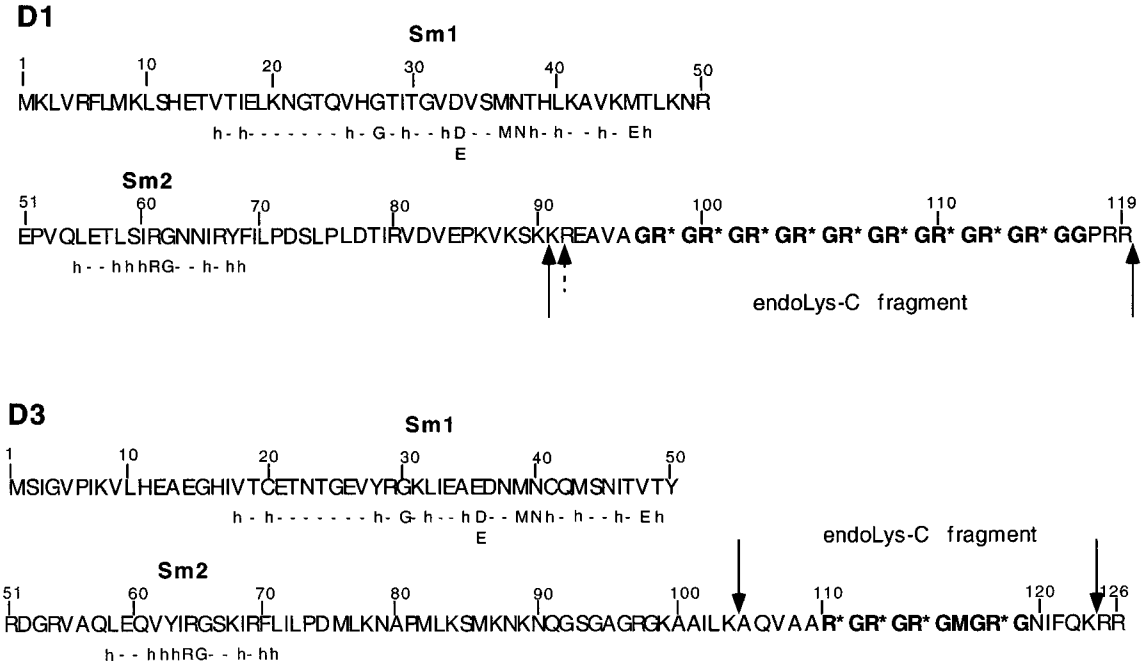

FIG. 1. **Location of sDMAs in the amino acid sequences of the Sm proteins D1 and D3.** The RG stretches are presented in *boldface type*, and sDMAs are indicated by *asterisks*. The position of the Sm 1 and Sm 2 motifs is shown by the consensus sequence *below* the protein sequences. Sequenced C-terminal D1 and D3 fragments obtained by proteolytic digestion with endo-Lys-C are indicated by *arrows*.

binant proteins were sprayed as lines on a nylon membrane with a plastic backing and treated as described previously (37). Patient sera and Y12 were applied in a dilution of 1:200. A 3-fold molar excess of biotinylated synthetic peptides in 50 mM sodium carbonate buffer (pH 9.6) were complexed to streptavidin by incubating for 1 h at 37 °C, before applying to the membrane. In order to visualize the applied proteins or peptides, a nonblocked LIA strip was stained with colloidal gold (Aurodye; Amersham Pharmacia Biotech) according to the manufacturer's procedure.

RESULTS

The Human Sm Proteins D1 and D3 Contain sDMAs at Their Carboxyl Termini—To investigate whether the RG-rich stretches of the human D1 and D3 proteins contain methylated arginines, we performed protein sequencing and mass spectrometry. HeLa UsnRNPs were initially purified by immunoaffinity chromatography with an H2O antibody directed against the m_3G cap of UsnRNPs (38), and the protein constituents of the human UsnRNPs were then fractionated by High-TEMED SDS-polyacrylamide gel electrophoresis (16). Subsequently, those bands containing the D1 and D3 proteins were excised from the gel and proteolytically digested with endo-Lys-C. This procedure resulted in a C-terminal 29-amino acid-long D1 fragment (amino acids 91–119) and a 23-amino acid-long D3 fragment (amino acids 105–124) (see Fig. 1). After fractionation of the D1 and D3 peptide mixtures by HPLC, we were able to isolate both C-terminal peptides, so that all C-terminal RG dipeptides of D1 and D3 were analyzed (Fig. 1).

To initially determine whether the C-terminal peptide of D1 contains posttranslational modifications, we performed mass spectrometry (MALDI-TOF). A protonated major mass peak, isotopically resolved at 3366.68 Da, was detected (Fig. 2). The observed value clearly deviated from the theoretical mass expected for a nonmodified D1-(91–119) peptide. Moreover, the mass difference fit almost perfectly for a D1 peptide with dimethylated arginines at all positions of its RG-rich stretch (the theoretical protonated monoisotopic value of D1-(91–119) with nine DMAs is 3366.10 Da). A minor peak of 3238.58 Da, obtained to a lesser extent after proteolytical digestion, was also observed and is consistent with a dimethylated D1-(92–119) peptide (Fig. 2). Two additional peaks of 3252.85 and 3380.65 Da corresponded to D1-(92–119) and D1-(91–119) peptides with an additional methyl group, due to esterification of the carboxyl termini upon storage in an acidified methanolic solution. The MALDI-TOF analysis of D1 thus not only demonstrated the presence of posttranslational modifications in the C terminus of D1, but also strongly suggested the presence of DMAs.

To confirm the results of our mass spectrometry experiments and to distinguish between asymmetrically and symmetrically dimethylated arginines (see Fig. 3), we analyzed the C-terminal D1 and D3 peptides by protein sequencing. For this purpose, we not only used the normal PTH-derivative standard during the sequencing run but additionally applied MMA, aDMA, and sDMA as a standard for the HPLC-based identification of amino acids. Fig. 3*A* shows an alignment of the Edman degradation cycles of the gel-purified C-terminal peptide of HeLa D1 with the HPLC elution profiles of PTH-modified MMA, aDMA, and sDMA (indicated by *arrows*). We obtained a clear separation of arginine, MMA, aDMA, and sDMA (Fig. 3, *A* and *B*). When we compared the elution profiles of the C-terminal peptide of HeLa D1 with the applied standards, we could reproducibly identify symmetrical DMAs in the C terminus of D1. As emphasized by the cycle numbers in Fig. 3*A* (only the cycles corresponding to the arginines are shown), sDMA is present at all nine positions in the RG-rich stretch of D1. Only the arginines at positions 2, 28, and 29 of the D1 peptide (positions 92, 118, and 119 of D1), which are not flanked by glycines, were not dimethylated (data not shown), thus providing evidence that the methyltransferase modifying D1 recognizes solely its RG repeat. In agreement with our mass spectrometry experiments (Fig. 2), we could not observe even minor peaks corresponding to arginine or MMA (see Fig. 3*A*). The absence of detectable amounts of undermethylated D1 precursors in HeLa nuclear extracts may hint at a cytoplasmic localization of the D1-modifying methyltransferase.

In the D3-derived peptide encompassing amino acid residues 105–124, four symmetrically methylated arginines at positions

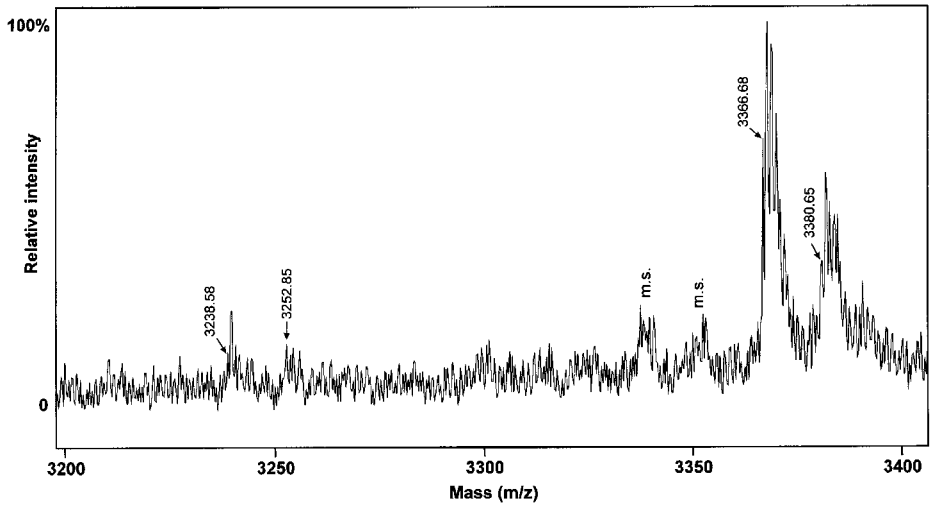

FIG. 2. **MALDI-TOF mass spectrum of an HPLC-purified fraction containing the modified arginine C-terminal peptide that was obtained by endo-Lys-C digestion of human D1.**

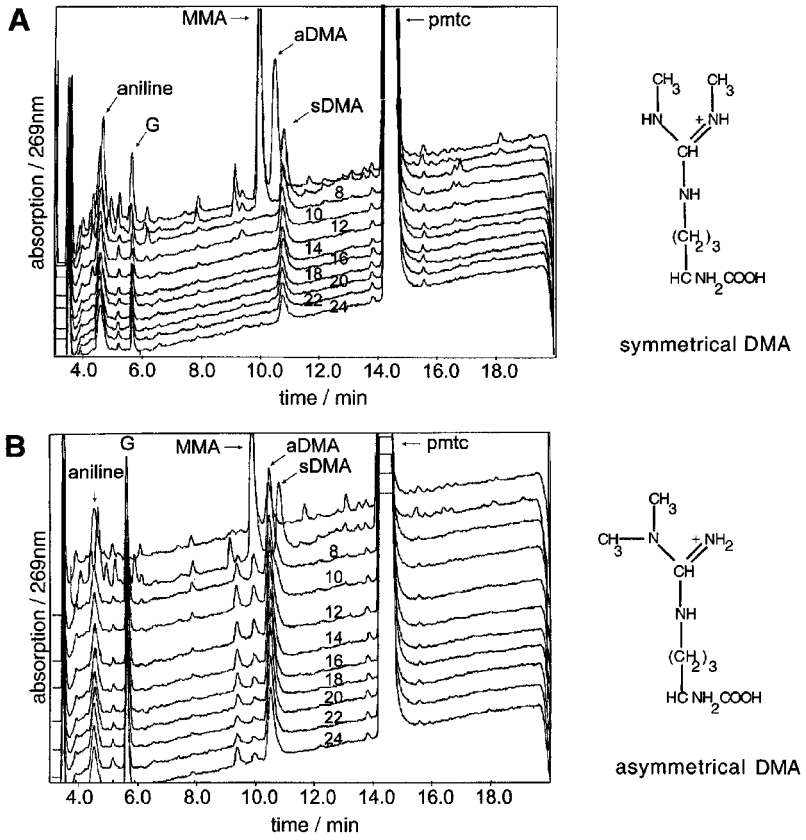

FIG. 3. *A*, PTH-derivative sequence analysis of an HPLC-purified fraction containing the C-terminal peptide generated by endo-Lys-C digestion of human D1. An alignment is shown of the Edman degradation cycles 8, 10, 12, 14, 16, 18, 20, 22, and 24 (corresponding to arginines in positions 98, 100, 102, 104, 106, 108, 110, 112, and 114 of D1). The elution profiles of MMA, aDMA, and sDMA are indicated by *arrows*. The structure of sDMA is shown on the *right*. *B*, PTH-derivative sequence analysis of an HPLC-purified fraction containing the C-terminal peptide generated by endo-Lys-C digestion of baculo-derived D1. The same Edman degradation cycles as in *A* are aligned. The structure of aDMA is shown on the *right*.

110, 112, 114, and 118 of the D3 protein were identified (data not shown). Interestingly, the sDMA at position 110 of D3, in contrast to all other modified arginines in the RG-rich stretches of D1 and D3, was not preceded by a glycine (alanine instead of glycine; see Fig. 1). This raised the possibility that such RG dipeptides generally could serve as substrates for posttranslational methylation. However, RG dipeptides present in the Sm motif 2 of D1 and D3 (amino acid 61 in D1 and 64 in D3; Fig. 1) were not modified. Also, methylated arginines were not found in the single RG dipeptides present in the Sm proteins E, F, G, and D2 (data not shown). As observed with D1, the sDMA precursors arginine and MMA were not detected in D3. These results thus demonstrated that, in contrast to several RGG box-containing proteins that contain asymmetrical DMA, the human Sm proteins D1 and D3 are symmetrically dimethylated. Moreover, since we isolated the D1 and D3 proteins from biochemically purified HeLa UsnRNPs, symmetrical dimethylation of D1 and D3 occurs *in vivo*.

Recombinant D1 from E. coli and Baculovirus-infected Insect Cells Is Only Poorly Recognized by Anti-Sm Patient Sera in LIAs—D1 overexpressed in *E. coli* has been reported to react only weakly and sometimes unspecifically with patient sera (see Introduction). Moreover, the prototypical anti-Sm antibody, Y12 (39), strongly reacted with native D1, D3, and B/B′ in immunoblots but not with recombinant D1 overexpressed in *E. coli* (Ref. 16 and data not shown). It was thus conceivable that the B-cell epitopes recognized by Y12, and by at least some anti-Sm autoantibody populations, require sDMA for efficient recognition. To investigate this hypothesis, we overexpressed D1 in either *E. coli* or in baculovirus-infected insect cells. For one-step purification by nickel affinity chromatography, both proteins were overexpressed as fusion proteins containing the

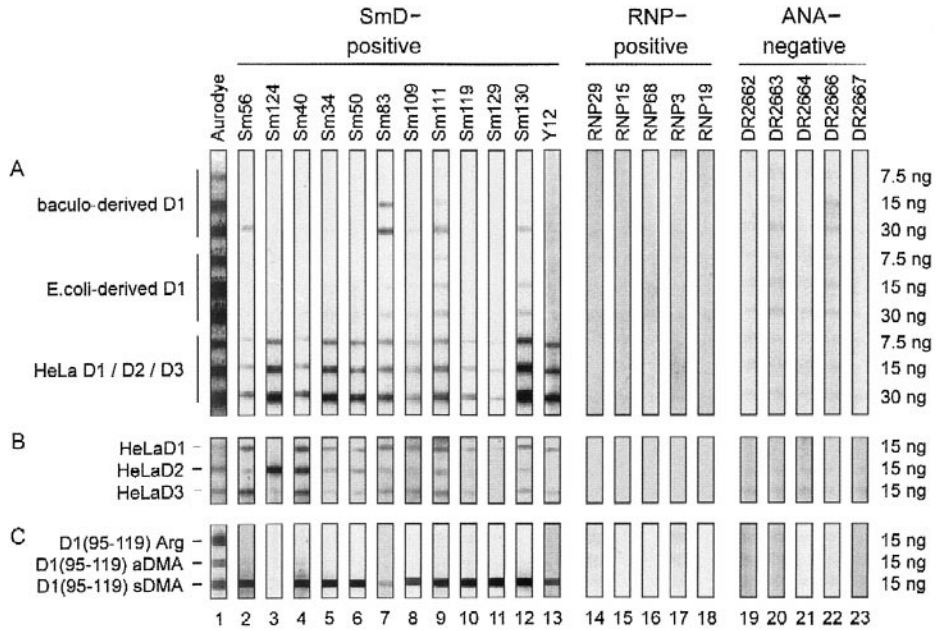

FIG. 4. **LIA with patient sera and different Sm antigen sources.** The strips were tested with 11 anti-Sm patient sera (*SmD-positive*), five anti-RNP sera from patients with mixed connective tissue disease (*RNP-positive*), and five control sera that were found negative for anti-nuclear antibodies (*ANA-negative*). The designations of the sera are indicated at the *top*. The antigen sources are indicated to the *left*. The amount of applied antigen is indicated to the *right*. *A*, baculo-derived D1, *E. coli*-derived D1, and a mixture of gel-purified human D1, D2, and D3 from HeLa cells were applied to the strips at a concentration of 7.5, 15, and 30 ng/line. *B*, 15 ng of gel-purified, human D1, D2, or D3 protein from HeLa cells were applied as antigen source. *C*, line immunoassay with synthetic peptides, encompassing the D1 amino acids 95–119. Peptide D1-(95–119) Arg contained nonmodified arginines instead of sDMA in the *in vivo* dimethylated positions. Peptide D1-(95–119) aDMA contained aDMA instead of sDMA. Peptide D1-(95–119) sDMA contained sDMA in the *in vivo* dimethylated positions. All peptides were applied to the membrane at a concentration of 15 ng/line.

first 25 amino acids of mouse tumor necrosis factor and six histidines (see "Experimental Procedures"). In electrospray mass spectrometry, we observed a mass of 17,435 Da for the *E. coli*-derived D1, which was the expected mass for the full-size fusion protein without any posttranslational modifications. In contrast, the purified D1 from the baculovirus expression system yielded several peaks with significantly higher masses (data not shown), thus providing evidence that posttranslational methylation of recombinant D1 had taken place. To further investigate the dimethylation status of the baculo-derived D1, we performed protein sequencing as described for the HeLa-derived D1 protein (see Fig. 3*B*). Although we could isolate a minor HPLC fraction containing nonmodified peptide D1-(91–119), the majority of the baculo-derived D1 was indeed dimethylated, as predicted by the mass spectrometry (see above). However, in contrast to the HeLa-derived D1 protein, the baculo-derived protein contained asymmetrical, as opposed to symmetrical, dimethylarginines (see Fig. 3*B*). The less prominent peaks preceding the aDMAs in the HPLC elution profiles in Fig. 3*B* co-eluted with the dimethylation precursors, arginine and MMA.

To analyze the immunological properties of the recombinant D1 proteins, we applied equal amounts of baculo-derived D1, *E. coli*-derived D1, and a mixture of gel-purified human D1, D2, and D3 (HeLa D1/D2/D3 in Fig. 4*A*), which served as a positive control, to nylon strips. The strips were then incubated with anti-Sm sera from SLE patients and the monoclonal anti-Sm antibody Y12. As negative controls, we used sera from patients with mixed connective tissue disease, containing high titers of anti-RNP autoantibodies against the U1-A, U1-C, and U1–70K proteins (RNP-positive in Fig. 4) as well as sera from healthy patients that did not contain antinuclear autoantibodies (ANA-negative in Fig. 4). While a mixture of D1, D2, and D3 from HeLa cells reacted significantly with all of the tested anti-Sm patient sera, the *E. coli*-derived D1 gave only weak signals, which were in the intensity range of signals obtained with control sera (compare D-positive sera with ANA-negative and RNP-positive sera in Fig. 4*A*). Interestingly, also the baculo-derived D1 reacted only weakly with anti-Sm patient sera; only four bands of significant intensity were observed with baculo-derived D1 (sera Sm56, Sm83, Sm111, and Sm130 in Fig. 4*A*). Note that similar amounts of antigen had been applied to the strips, as controlled by aurodye staining (Fig. 4*A*, *lane 1*). To verify that the tested anti-Sm patient sera contained anti-D1 autoantibodies, we additionally tested the patient sera with single HeLa-derived D1, D2, and D3 proteins in LIA (Fig. 4*B*). Except for serum Sm124, which reacted exclusively with D2 (Fig. 4*B*, *lane 3*), all anti-Sm sera reacted significantly with HeLa-derived D1, including those that did not react with baculo-derived D1 (compare, for example, Sm34; *lane 5* in Fig. 4, *A* and *B*). Although we cannot fully exclude the possibility that the weak antigenicity of the recombinant D1 proteins was due to the extra amino acids of the fusion region, the less frequent recognition of both *E. coli*-derived and baculo-derived D1 indicated that the presence of sDMAs strongly increases the antigenicity of the D1 protein.

Anti-Sm Patient Sera Specifically Recognize the sDMA-containing Carboxyl Terminus of D1—The predominant recognition of HeLa-derived D1 in LIA strongly suggested that the symmetrically dimethylated C terminus of D1 forms a B-cell epitope for anti-Sm autoantibodies. To analyze this, we chemically synthesized peptides containing nine aDMAs, nine sDMAs, or nine arginines in the *in vivo* methylated positions and used them as antigens in LIA experiments with the aforementioned patient sera. All peptides were purified by HPLC and characterized by mass spectrometry. The presence of sDMA was demonstrated by sequencing. To ensure efficient binding of the peptides to the membrane, we conjugated them with an N-terminal biotin residue during peptide synthesis and formed a streptavidin-peptide complex before applying the antigens to

the membrane. Indeed, the complexed peptides bound well to the membrane (Fig. 4*C*, *lane 1*). As shown in Fig. 4*C*, 10 of 11 anti-Sm patient sera significantly reacted with the sDMA-containing peptide. In contrast, peptides with either arginine or aDMA were not recognized by anti-Sm patient sera. These data not only demonstrate that anti-Sm autoantibody populations frequently recognize the C terminus of D1 directly but also show that the efficient recognition of this epitope is dependent on the presence of sDMA. Since sera from mixed connective tissue disease patients with high titers of anti-RNP autoantibodies and control sera from healthy patients did not react with the sDMA-containing peptide, the reaction was SLE-specific. The specificity of the LIA was further substantiated by the finding that the only anti-Sm patient serum that did not react with the peptide was a serum that exclusively reacted with D2 (see Fig. 4, *B* and *C*, *lane 3*). The negative signal observed with this serum was thus due to the absence of anti-D1 autoantibodies. Strikingly, the monoclonal antibody Y12 also reacted with the peptide D1-(95–119) sDMA, but not with D1-(95–119) Arg or D1-(95–119) aDMA (Fig. 4*C*, *lane 13*). Previous studies using synthetic peptides or *E. coli*-derived deletion mutants had always failed to generate significant signals with this antibody (see "Discussion"). Our results indicate that Y12 also requires sDMA for antigen recognition, at least in the epitope localized on D1. Moreover, since an sDMA-containing RG-rich stretch is also present in the D3 protein, an epitope in this region can easily explain the cross-reactivity of Y12 and anti-Sm autoantibodies with D1 and D3.

DISCUSSION

In this paper, we demonstrate by protein sequencing and mass spectrometry that the human Sm proteins D1 and D3 are symmetrically dimethyated at C-terminal arginines *in vivo*. This is the first time that posttranslational modifications have been localized in Sm proteins. The presence of the unusual amino acid sDMA in D1 and D3 could have implications both for snRNP biogenesis and SLE etiology.

The elucidation of the antigenic determinants recognized by anti-Sm autoantibodies is of considerable interest to understand and diagnose the autoimmune disease SLE (see Introduction). In antibody binding studies, we could clearly identify a linear epitope for anti-Sm autoantibodies and the monoclonal antibody Y12 on the amino acids 95–119 of the human D1 protein. Importantly, the intensities of the signals observed with the D1 peptide 95–119 containing sDMA were in the range of the signals obtained with the native D1 protein (Fig. 4, compare *B* and *C*). Previous studies have also mapped an epitope for anti-Sm autoantibodies at the C terminus of D1 (23, 27–29, 40). However, in these studies, the analyzed D1 fragments did not contain sDMA and were not compared with a wild type protein, raising the possibility that the binding of anti-Sm autoantibodies was only weak. Indeed, in our hands, a nonmodified D1 peptide 95–119 and an aDMA-containing peptide 95–119 showed only basal level signal intensities, as compared with the native D1 protein (Fig. 4). Our data thus demonstrate that the presence of sDMA strongly increases the affinity of anti-Sm autoantibodies for the C terminus of D1.

The sDMA-containing peptide D1-(95–119) is the first synthetic peptide found to significantly react with the prototypical anti-Sm antibody Y12 (Fig. 4*C*). Deletion mutagenesis, using *in vitro* translated proteins, has located the Y12 epitope to the C termini of the Sm proteins D1 and B/B′ (24, 41) and the ribosomal S10 protein (42), all of which contain at least one GRG tripeptide. However, neither synthetic peptides encompassing the mapped epitopes nor *E. coli*-derived D1, D3, or B/B′ reacted significantly with Y12 (24).[2] Our data therefore suggest that symmetrically dimethylated RG stretches constitute a major Y12 epitope. The dimethylation status of B/B′ or S10 is not yet clear; however, the presence of GRG tripeptides in the mapped Y12 epitopes of these proteins raises the possibility that they may also contain sDMA. However, although protein sequencing of single RG dipeptides in the E, F, and G proteins revealed that these proteins are not dimethylated (data not shown), an E·F·G complex is significantly immunoprecipitated by Y12 (18). This indicates that Y12 may recognize more than one epitope on the Sm proteins.

The high efficiency by which the sDMA-containing peptide is recognized by anti-Sm autoantibodies and Y12 demonstrates that the C-terminal, linear epitope contributes significantly to the antigenicity of the full-length human D1 protein. Accordingly, the *E. coli*-derived D1, which contained nonmodified arginines was not recognized by anti-Sm autoantibodies (Fig. 4, compare *A* and *C*). However, 4 of 11 anti-Sm sera significantly reacted with the aDMA-containing baculo-derived D1 but not with the aDMA-containing peptide. This indicates that the baculo-derived D1 contains additional epitopes, *e.g.* discontinuous epitopes on the Sm domain (see Introduction), that were recognized by anti-Sm autoantibodies. Ou *et al.* (32) reported that 96% of anti-Sm patient sera recognized recombinant D1 overexpressed in baculovirus-infected insect cells in enzyme-linked immunosorbent assays, while 71% of patient sera recognized D1 overexpressed in *E. coli*. The increased antigenicity of the baculo-derived D1 as compared with the *E. coli*-derived D1 suggests that the asymmetrical dimethylation of D1 in the former case stabilizes conformational epitopes on the Sm domain. The same may be true for the symmetrical dimethylarginines. The lower frequency of recognition of the recombinant D1 proteins in our LIA as compared with the study of Ou *et al.* could therefore be due to the increased degree of renaturation in the enzyme-linked immunosorbent assay protocol used by Ou *et al.* (Ref. 32; see also Ref. 40). However, since both the HeLa-derived D proteins and the peptide D1-(95–119) sDMA were recognized with a high frequency (10/11) and specificity by patient sera in our study, native Sm proteins and the sDMA-containing peptide are interesting substrates for the diagnosis of SLE. Studies to investigate the specificity and sensitivity of anti-Sm autoantibody detection in an extended SLE patient population, using the peptide D1-(95–119) sDMA as an antigen, are currently under way.

Little is known about the etiology of SLE and other autoimmune diseases. We have demonstrated that symmetrical dimethylation can increase the affinity of anti-Sm autoantibodies toward their antigenic target. Similarly, a class of rheumatoid arthritis-associated autoantibodies (APF/AKA) reacted exclusively with citrulline-containing peptides of the rheumatoid arthritis autoantigen filaggrin but not with homologous arginine-containing peptides (43, 44). Autoantigens containing asymmetrically dimethylated arginines have also been described: fibrillarin in scleroderma (45), heterogeneous nuclear ribonucleoprotein A1 in connective tissue diseases (46), and nucleolin in SLE (47). It is therefore tempting to speculate that posttranslational modifications might play a role in the etiology of SLE and other autoimmune diseases. Recently, much attention has been paid to posttranslational protein modifications during apoptotic processes, which may lead to the subsequent development of autoantibodies (48). Abnormal apoptosis, such as observed in SLE (for a review, see Ref. 49), could result in the continuous presentation of modified autoantigens that are normally retained in the nucleus (50). During apoptosis,

[2] H. Brahms and R. Lührmann, unpublished observations.

nuclear, dimethylated D1 could induce an autoimmune response in an antigen-driven manner. To investigate this hypothesis, it will be of considerable interest to analyze the immunogenicity of the dimethylated C terminus of D1 in animal models.

The D1 and D3 proteins are the first examples of human nuclear proteins that are symmetrically dimethylated *in vivo*. Other dimethylated nuclear proteins contain asymmetrical DMAs (see Introduction). Indeed, the only other human protein shown to contain sDMA to date is MBP, which is a major component of the nerve sheath and implicated in the autoimmune disease multiple sclerosis (51). This raises the question of which protein arginine *N*-methyltransferase is responsible for the methylation of the D1 and D3 proteins. Type I methyltransferase, which was recently cloned from yeast (52), rat (53) and humans (54), catalyzes the formation of aDMAs in histones and heterogeneous nuclear ribonucleoprotein A1 (for a review, see Ref. 31). In contrast, the gene for the type II methyltransferase that specifically modifies MBP to create sDMAs has not yet been identified. The symmetrical dimethylation of D1 and D3 would suggest that these proteins are substrates for a type II methyltransferase. MBP contains only a single sDMA at position 107 (51), and the amino acid sequence surrounding Arg-107 (^{104}GKGRGL^{109}) slightly resembles the D1 RG repeat. Interestingly, a heptapeptide with alternating glycines and arginines (GRGRGRG), such as found in D1, was the most effective methyl acceptor for a partially purified MBP-specific type II protein methyltransferase (55). However, only MMA with a minor fraction of aDMA was formed *in vitro* with this peptide, providing evidence that methyltransferases may change their activity dependent on the substrate. It is therefore possible that proper dimethylation of D1 and D3 requires a particular hetero-oligomeric protein environment, *e.g.* assembled core UsnRNPs or Sm protein complexes (see Introduction). Proper assembly as a prerequisite for proper dimethylation could easily explain our observation that baculo-derived D1 contained aDMA instead of sDMA (see Fig. 3*B*), since the overexpressed D1 protein does not come in contact with stoichiometric amounts of the other Sm proteins or UsnRNA to form the D1·D2 complex or core UsnRNPs. To unambiguously identify and characterize the D1/D3-modifying enzyme, detailed *in vitro* methylation studies with recombinant and/or purified methyltransferases and with different substrates (such as the heteromeric protein complexes involved in Sm core formation) will be necessary.

The biological function of arginine methylation is largely unknown. The strongly increased binding of Y12 and anti-Sm autoantibodies to D1 in the presence of sDMA suggests that the methylation of the D1 and D3 proteins may modulate interactions with other cellular components. In the case of MBP, the sDMA at position 107 is indeed involved in the integrity and compactness of myelin. Moreover, it has been demonstrated that the MBP-specific methylase activity occurs concurrently with the myelination process (for a review, see Ref. 56). Likewise, the methylation of D1 and D3 could stabilize or weaken protein-protein or protein-RNA interactions, thus regulating the biogenesis of UsnRNPs. The formation of complexes between the Sm proteins (see Introduction) is probably not affected by arginine methylation, since the yeast D1 and D3 homologues, which form equivalent complexes, lack an RG-rich stretch (57, 58). In addition, it has been demonstrated for the human Sm proteins that the Sm domain alone is sufficient for complex formation (3, 22). However, arginine methylation could be involved in the binding of the Sm core to UsnRNP-specific proteins (12) or could reduce unspecific protein-RNA interactions of D1 and D3 by steric hindrance (59). It has also been suggested that methylated arginines may facilitate transport events (60). Since one part of the bipartite nuclear localization signal of the UsnRNPs is situated on the core proteins (10, 11), it is possible that arginine methylation facilitates the binding of the properly assembled core-UsnRNP to a still unidentified nuclear import factor. Consistent with this possibility, our data suggest that methylation of D1 takes place in the cytoplasm, since all D1 molecules we isolated from nuclear extracts were completely dimethylated. If methylation were to occur in the nucleus, minor amounts of unmodified arginine and MMA would be expected at positions 98–114 of D1. To understand the biological function of the symmetrical dimethylation of the D1 and D3 proteins, it will therefore be of considerable interest to analyze the cytoplasmic assembly intermediates of the UsnRNPs for dimethylated arginines.

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PROTEIN SYNTHESIS
POST-TRANSLATION MODIFICATION
AND DEGRADATION:

The C-terminal RG Dipeptide Repeats of the Spliceosomal Sm Proteins D1 and D3 Contain Symmetrical Dimethylarginines, Which Form a Major B-cell Epitope for Anti-Sm Autoantibodies

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