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Monoclonal Antibody Specific to a Subclass of Polyproline-Arg Motif Provides Evidence for the Presence of an snRNA-Free Spliceosomal Sm Protein Complex In Vivo: Implications for Molecular Interactions Involving Proline-Rich Sequences of Sm B/B′ Proteins

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Abstract The human spliceosomal Sm B/B′ proteins are essential for the biogenesis of the snRNP particles. B/B′ proteins contain several clusters of the PPPPGM/IR sequence, which occurs within the C-terminus of Sm B/B′. This sequence is very similar to the PPPPPGHR sequence of the cytoplasmic tail of the CD2 receptor and closely resembles the class II of SH3 ligands, suggesting a similarly important role. We report that a monoclonal antibody (3E10) against the PPPPPGHR sequence recognizes spliceosomal Sm B/B′ proteins. Proteins that are specifically immunoprecipitated by 3E10 include Sm B, B′, D1, D2, D3, E, F, and G. However, unlike Y12 and other anti-Sm immunoprecipitates, 3E10 immunoprecipitates appear to lack the U1 snRNP-specific proteins A and C and U snRNAs. These findings indicate that 3E10 recognizes a subset of Sm protein core and suggest the presence of snRNA-free Sm protein complex(es) in vivo. We propose that the epitope binding for 3E10 may become unaccessible upon interactions of Sm proteins and their subsequent incorporation into the core particles. The Sm proline-rich sequences may have an important role in mediating protein-protein interactions necessary for the proper snRNP core assembly or function, or both. To our knowledge, 3E10 is the first well characterized mAb specific for a subclass of polyproline-arg motif recognizing Sm B/B′ and CD2 proteins. 3E10 antibody can be used to further characterize the nature of protein components in the snRNA-free Sm subcore protein complex(es) that are formed during the snRNP core assembly steps. J. Cell. Biochem. 74:168–180, 1999. © 1999 Wiley-Liss, Inc.

Key words: monoclonal antibody; proline-rich motif; Sm B/B′ proteins; CD2

The small nuclear ribonucleoproteins (snRNPs) U1, U2, U4/U6, and U5 are major constituents of the spliceosome and are important in the biogenesis of U snRNPs, the catalytic center of the pre-mRNA splicing reaction [Moore et al., 1993, and references therein]. Their proteins are classified into two categories: the common proteins called Sm proteins, which are shared by the four snRNPs, and the snRNP specific proteins. The U1 snRNP contains three

Abbreviations used: snRNPs, small nuclear ribonucleoproteins; NLS, nuclear localization signal; SDS-polyacrylamide gel electrophoresis, SDS-PAGE; polyvinylidene difluoride membrane, PVDF; two-dimensional isoelectric focusing gel electropheresis, 2D-PAGE; two-dimensional nonequilibrium pH gradient gel electropheresis, NEPHGE.

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specific proteins designated 70k, A, and C. The other snRNP particles contain more than 10 specific proteins each. These specific proteins are thought to mediate snRNP particle-specific functions in pre-mRNA splicing [Lührmann et al., 1990]. The core structure of snRNPs consist of at least eight Sm proteins, termed B′ (29 kD), B (28 kD), D3 (18 kD), D2 (16.5 kD), D3 (16 kD), E (12 kD), F (11 kD), and G (9 kD) bound to appropriate RNA [Lührmann et al., 1990]. All the cDNAs encoding for the human core proteins have been cloned [Lehmeier et al., 1994]. With the exception of B and B′, all core proteins are encoded by separate genes; the former are produced by alternative splicing of a single gene product and differ by only 11 amino acids at the C-terminus [Chu et al., 1991; Van Dam et al., 1989].

The Sm core proteins are important for several reasons. First, they are essential for the biogenesis of the snRNP particles. The biogenesis of snRNPs is a complex multistep process that requires the bidirectional transport of snRNAs across the nuclear envelope [DeRobertis, 1983; Mattaj, 1986; Neuman et al., 1990]. The snRNAs are transcribed in the nucleus by RNA polymerase II, whereas the Sm proteins are synthesized in the cytoplasm and do not migrate on their own into the nucleus. Instead, the snRNA are exported from the nucleus to the cytoplasm where the Sm proteins bind to the snRNA Sm site to form the Sm core. Association of the Sm proteins with the Sm site is essential for the hypermethylation of the snRNA cap structure to generate the m_3G cap [Mattaj et al., 1986; Fischer et al., 1990, 1993; Hamm et al., 1990]. In addition, the core proteins play an important role in the formation of one part of the bipartite nuclear localization signal (NLS) of the snRNP particle; the m_3G cap forms the second part of the NLS [Fischer et al., 1990, 1993].

Formation of the Sm core domain in the cytoplasm requires specific interactions among Sm proteins to allow their binding to the Sm of newly transcribed U snRNAs [Fisher et al., 1985; Sauterer et al., 1990; Feeney et al., 1989; Raker et al., 1996]. Proteins E, F, and G and one or more of the D proteins are thought to assemble independently of snRNA into a heterooligomeric protein complex. The binding of the Sm protein complex to the Sm site results in the formation of a subcore, which is then completed to the mature Sm core by the addition of a complex of B, B′, and D3 proteins [Raker et al., 1996]. Recent studies have indicated that these assembly steps are assisted by additional non-snRNP factors [Liu et al., 1997; Fischer et al., 1997]. However, the nature of the protein-protein interactions in the core Sm structure of the snRNP is not yet understood, and the detailed mechanism of the way in which the Sm core proteins and the snRNP-specific proteins form functional assembled snRNPs is unclear.

The Sm core proteins are also interesting from a clinical and immunological point of view. Patients suffering from systemic lupus erythematosus (SLE), a multisystem rheumatic disorder, often produce anti-Sm antibodies that react predominantly with B and D proteins [Habets et al., 1989; Lerner et al., 1981; Elkon et al., 1990; James et al.., 1992, 1995]. These autoantibodies precipitate snRNP containing the U1, U2, U4/U6, and U5 RNA. Why the spliceosome is a major target of SLE autoimmunity is unknown.

The C-terminus of Sm B proteins is characterized by the presence of several repeats of the sequence PPPG(I/M)R [James et al., 1992, 1995]; these sequences were previously shown to be immunoreactive in Sm B/B′ [James et al., 1992, 1995]. The role of the repeated proline-rich motif in Sm B/B′ proteins is unknown. Interestingly, these proline-rich repeats are similar to proline-rich motifs found on proteins that associate with SH3 domains, such as the one found in the cytoplasmic tail of the CD2 receptor [Kamoun et al., 1981; Chang et al, 1990]. The repeated proline-rich domains are important for CD2 signaling and are able to mediate binding to SH3-containing proteins [Bell et al., 1996; Lin et al., 1998]. The SH3 domain mediates protein-protein associations through the recognition of proline-rich motifs on associated proteins and is important for coupling of intracellular signaling pathways, regulation of catalytic activity of proteins, recruitment of substrates to enzymes, and localization of proteins to a specific subcellular compartment [Cicchetti et al., 1992; Egan et al., 1993; Gout et al., 1993; Catling et al., 1995].

We report that a monoclonal antibody (3E10) raised against the proline-rich sequence (PPPPPGHR) of the cytoplasmic tail of the CD2 receptor recognizes spliceosomal B/B′ proteins. This observation strongly suggests that the PPPPG(M/I)R sequence present in the Sm B/B′ is structurally and immunologically similar to

the PPPPGHR sequence of the cytoplasmic tail of CD2, which plays an important role in signal transduction. This study showed interesting features about the epitope recognized by monoclonal antibody (mAb) 3E10 and provided evidence for the presence of snRNA-free Sm protein complex(es) in Jurkat cell extracts. In addition, this finding strongly suggests that the proline-rich epitope recognized by mAb 3E10 may become unaccessible upon interactions of Sm proteins and/or their subsequent incorporation into the U snRNP particle. Thus, PPPPG (M/I)R sequences of Sm B/B′ may mediate protein-protein interactions important for the assembly and/or targeting of specific snRNP core proteins to specific subcellular compartment.

To our knowledge, 3E10 is the first well-characterized mAb specific for a subclass of polyproline-arg motif recognizing Sm B/B′ and CD2 proteins. The 3E10 antibody can be used to further characterize the nature of protein components in the snRNA-free Sm subcore protein complex(es) formed during the snRNP core assembly steps.

MATERIALS AND METHODS

Cell Culture and Treatments

The human T-leukemic Jurkat cell line (J32) and its $CD2^-$ mutant J32–3.2 were previously described [Makni et al., 1991]. Cell lines were cultured in RPMI-1640 medium (Life Technologies, Grand Island, NY), supplemented with 10% (v/v) prescreened heat inactivated FCS and 2 mM L-glutamine. Cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2. For in vivo labeling with a [^{35}S]cysteine-methionine mixture, J32 cells previously grown at low concentration (2×10^5/ml) were incubated with 50 μCi/ml of ^{35}S-[cys/met] Pro-mix (Amersham, IL) in cysteine- and methionine-free RPMI-1640 supplemented with 5% dialyzed FCS for 4–5 h at 37°C.

Preparation of Tetanus Toxoid- and BSA-Peptide Conjugates

Peptides were synthesized using an applied Bio-systems instrument and FMOC chemistry. The peptides were purified by high-performance liquid chromatography (HPLC) and checked by sequencing (Cancer Center Core Biochemistry Laboratory Facility, University of Pennsylvania Medical Center, Philadelphia, PA). Tetanus toxoid- and bovine serum albumin (BSA)-peptide conjugates were prepared as follows: CD2-P1 starting with a N-terminal cysteine and consisting of amino acids 277–291 (ATSQHPPPPPGHRSQ) of the cytoplasmic region of the human CD2 molecule, was cross-linked to tetanus toxoid or BSA using sulfo-SMMC (Pierce) as a cross-linker. The cross-linked tetanus toxoid- and BSA-conjugates were separated from unreacted peptides and cross-linking reagent by dialysis against PBS pH 7.4 and concentrated with Centricon concentrator (Millipore).

Preparation of Monoclonal Antibodies

mAbs 3E10 (IgG1), 3G12 (IGg1), 3D12, 1A8.1B, and 1B2E7 were prepared by immunizing BALB/c mice with CD2-P1 conjugated to tetanus toxoid. Hybridoma cells were obtained by fusing spleen cells from immunized mice and SP2 myeloma cells as previously described [Kamoun et al., 1981]. Hybridoma were screened against the CD2-P1 conjugated to BSA by enzyme-linked immunoassay (ELISA), using goat anti-mouse-HRP as a secondary antibody. Anti-CD2 (9.6) was originally in our laboratory [Kamoun et al., 1981]. The anti-Sm (Y12) was provided by Dr. G. Dreyfuss and was originally a gift from Dr. J. Steitz [Lerner et al., 1981].

Immunoprecipitation of Cell Lysates

For immunoprecipitation, cells were resuspended in lysis buffer (50 mM HEPES, 150 mM NaCl, 10% glycerol, 1% Triton X100, 0.5% NP40, 20 mM EDTA, 8 μg/ml aprotinin, 200 μM phenylmethyl sulfonyl fluoride, 10 mM NaF, 10 mM sodium pyrophosphate, 2 μg/ml of leupeptine, and 200 μM sodium orthovanadate. Cell lysates were cleared by centrifugation at 12,000 rpm for 20 min, and the supernatants were added to 50 ml of Protein G-Sepharose (50% slurry) and gently mixed for 1 h at 4°C as a preclearing step. After the beads were spun, the supernatants were transferred to a new 1.5-ml Eppendorf tube containing antibodies bound to protein G-Sepharose (Pharmacia). The mixture was incubated with constant shaking for 1 h at 4°C and subsequently washed four times with the lysis buffer, followed three times with phosphate-buffered saline (PBS). High-stringency washes of the immunoprecipitates were performed once with 0.6 M NaCl, 12.5 mM KH_2PO_4, followed with another wash with mixed detergents including 0.5% Nonidet-40 (NP-40), 1% sodium dodecyl sulfate (SDS), 0.3 M NaCl, and

10 mM Tris-HCl pH 8.6. The proteins in the immunoprecipitates were eluted from the beads either with boiling in 2× Laemmli SDS buffer or vortexing the final pellet in the urea loading buffer (9.5 M urea, 2% NP-40, and 4% Ampholytes pH 3–10 [ESA Inc., MA]) mixture. The resulting samples were then analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) or two-dimensional isoelectric focusing gel electropheresis (2D-PAGE).

Western Blotting

The protein content of the cell lysate was determined with Bradford microassay (Bio-Rad, La Jolla, CA). A total of 20 μg of the total proteins from the cell lysate or immunoprecipitates was run on a 10–12% polyacrylamide gel containing 0.2% SDS and then transferred to a polyvinylidene difluoride membrane (PVDF) (Bio-Rad) in 0.5× Towbin buffer. The membrane was then quickly washed with 1× PBS containing 140 mM NaCl, 25 mM KCl, 1.5 mM NaH_2PO 4, 8 mM NaH_2PO_4, and 0.1% Tween-20, then blocked in the wash buffer containing 3% BSA, at +4°C overnight. The membrane was then incubated for at least 2 h in a plastic pouch containing 12 ml of the primary antibody diluted in the wash buffer containing 2% BSA. The membrane was then washed four times. Antigen-antibody complexes were detected by adding 0.5 μCi/ml of ^{125}I-Protein A or ^{125}I-goat anti-mouse IgG to the membranes, under agitation for 1 h in a plastic pouch. The membrane was washed extensively, partially dried and exposed to the x-ray film at −70°C for 1–3 days.

Purification of p16 Proteins and Peptide Microsequencing

The p16 protein was purified from J32 cells, using a single-step affinity antibody column (mAb 3E10), followed by a preparative SDS-polyacrylamide gel electrophoresis (PAGE). Soluble protein lysate was prepared from a large-scale culture of J32 cells (20 × 10^9) by sonication in 100 ml of lysis buffer containing a standard mixture of protease inhibitors without adding detergents. The lysate was clarified by centrifugation at 47,000 rpm for 2 h in a 50.2 Ti rotor (Beckman), and the supernatant was added to 2 ml of protein G-Sepharose beads for 1 h at 4°C as a preclearing step. The supernatants were transferred to a new tube containing 3 ml of protein G-Sepharose beads that was covalently cross-linked to 9 mg of affinity-purified mAb 3E10, using dimethyl pimelimidate. This method of mAb coupling provides maximum binding capacity, since the antibody on the column is theoretically oriented with all of its antigenic binding sites accessible to antigen. The precleared lysate was incubated with the beads for 2 h at 4°C. The beads were washed four times with 20 mM HEPES (pH 7.4), 150 mM NaCl, 10 mM EDTA, 1% Triton X-100, 0.5% NP-40, and the bound proteins were eluted by boiling in 1 ml of 50 mM Tris (pH 7.5), 2% SDS, 2 M β-mercaptoethanol and 5% glycerol. Sample is then concentrated using a SpeedVac concentrator to a final volume of 0.6 ml. The eluted proteins were loaded onto a 12% SDS-PAGE and resolved by electrophoresis. The resolved proteins were transferred to a high retention PVDF membrane (Bio-Rad) in Towbin 0.5× transfer buffer containing 10% MeOH, and the membrane was stained using 0.5% p/v Amido Black. Peptide microsequencing was performed at the Wistar Institute Protein Microchemistry Laboratory (Wistar Institute, Philadelphia, PA). Briefly, a single 16-kDa protein band (8 mg) was excised from the membrane, washed extensively with water, and incubated with trypsin. The cleaved peptides were resolved by reverse-phase HPLC. The three best-resolved peaks were analyzed by microsequencing using a gas-phase sequencer (Applied Bio-systems model 470). The following peptide sequences were determined: peptide 62A, AQVAAG-G-G-MG-GN-FQA. Cycles 6, 8, 10, and 14 in this sequence indicated that the same non common amino acid derivative at all four positions was observed. We were unable to identify this derivative after consulting several database sources. Mass Spectroscopy analysis indicated that the mass of this derivative is 184 Da; peptide 62B, HCNMVLENVK; peptide 64A, VLHEAEGHI-VTCETNTGEVYR; peptide 64A, LSHETV-TIELK.

Plasmid DNA In Vitro Translation

The clone encoding the Sm B protein was previously described [Raker et al., 1996]. In vitro translation of ^{35}S-labeled proteins was carried out using a combined transcription and translation kit (TnT) (Promega, Madison, WI) according to the instructions of the manufacturer. The in vitro translation products were then used for immunoprecipitation.

Two-Dimensional Gel Electrophoresis

The proteins, recovered in the urea loading buffer, were separated in the first dimension gel. For the two-dimensional nonequilibrium pH gradient gel electropheresis (NEPHGE), the upper chamber of the tank was filled with 0.1 M H_3PO_4 and the lower chamber was filled with 0.1 M NaOH. The samples and the pH gradient gels were run under 400 V for 15 h. The rod gels were equilibrated in the SDS buffer for 15 min before being subjected to the second dimension. For the second dimension, proteins were separated by SDS-PAGE. This was done by transferring the rod gels onto the top of the stacking gel, separating gels were 10–12.5%, 1.5 mm thick, acrylamide slab gel, as previously described [Kamoun et al., 1981]. After electrophoresis, the gels were fixed and treated with Amersham "Amplify" for fluorography as recommended by the manufacturer. To improve reliability and the reproducibility of the patterns, all the gels to be compared were run at the same time.

Sucrose Density Gradient Fractionation of Nucleoplasma and Isolation of U1 snRNP

Cells were labeled with 100 μCi/ml [^{35}S] methionine/cysteine as described above. Nucleoplasma from labeled cells were made as described [Pinol-Roma et al., 1990]. Cell pellets were resuspended in RSB-100 (10 mM Tris, 100 mM sodium chloride, 1 mM magnesium chloride, 2 mM DTT, and 1% Triton X-100) and sonicated for 10 s, 4 times at full blast power setting. Sonicated suspension was layered at the top of 2 ml 30% sucrose, and centrifuged at 3,000*g* for 15 min at 4°C. Top layer was collected, and 1 ml of nucleoplasma was layered at the top of 36 ml of 5–30% sucrose density gradient in polymer centrifuge tubes (Beckman). Sucrose gradient 5–30% was prepared in RSB-100, using BioComp, model 106 (New Brunswick, Canada). Polymer tubes were then ultracentrifuged in Beckman SW-28 rotor at 25,000 RPM for 42 h at 4°C. Thirty fractions, 1 ml each, were collected from top at 4°C using BioComp gradient fractionator model 150. Fractions sediments at 12 s are rich in U1 snRNP. These fractions were positively identified for U1 snRNPs by immunoprecipitation analysis using anti-RNP monoclonal antibodies [O'Connor et al., 1997].

Immunoprecipitation of RNA-Protein Complexes

Immunoprecipitation of RNA-protein complexes were performed as previously described [Fischer et al., 1993]. Briefly, immunoprecipitates from J32 total cell extract were washed with 70% ethanol, resuspended in H_2O, and labeled with the [^{32}P]pCp (0.1 μCi/ml) nucleotide, using the T4 RNA ligase, overnight on ice, the snRNA were re-extracted with the phenol, re-precipitated with ethanol and resuspended in 20 μl of the loading buffer. The RNA from each sample was applied on a 10% Sequagel.

RESULTS

Immunoprecipitates of 3E10 Contains Spliceosomal Sm Proteins

In order to better define the structural nature of the polyproline-arginine sequence found in the CD2 molecule as well as in the spliceosomal snRNP proteins, we raised several mAbs against the peptide sequence ATSQHPPPPP-GHRSQ. One of these antibodies (3E10) was fully characterized in a separate study using a phage-display library. mAb 3E10 was found to be specific for a subclass of polyproline-arginine motif PXXPP(G/X)XR wherein the Arg at the C-terminus appear to be the preferred flanking sequence (manuscript in preparation). To investigate the nature of proteins that are immunoprecipitated with mAb 3E10, we carried out immunoprecipitations using 3E10 from ^{35}S-labeled J32 cell lysates and the immunoprecipitated proteins were analyzed by SDS-PAGE. As shown in Figure 1A, several protein bands can be specifically immunoprecipitated with mAb 3E10. These proteins migrated with an apparent molecular weights of 16, 28/29, 46, 69, 97, and 160/180 kD. These proteins were not seen with the control antibody (9.6). The presence of the 16-kD, 28/29-kD proteins in 3E10 immunoprecipitates is not dependent on the expression of the CD2 molecule, as similar SDS-PAGE patterns of 3E10 immunoprecipitates were obtained with the $CD2^-$ mutant cell line J32–3.2, including the presence of the 28/29-kD and 16-kD bands (data not shown).

In order to further identify the nature of the proteins in the 3E10 immunoprecipitate, we purified some of these proteins in sufficient quantities for peptide microsequencing. The protein purification strategy was based on our observation that these proteins are present in

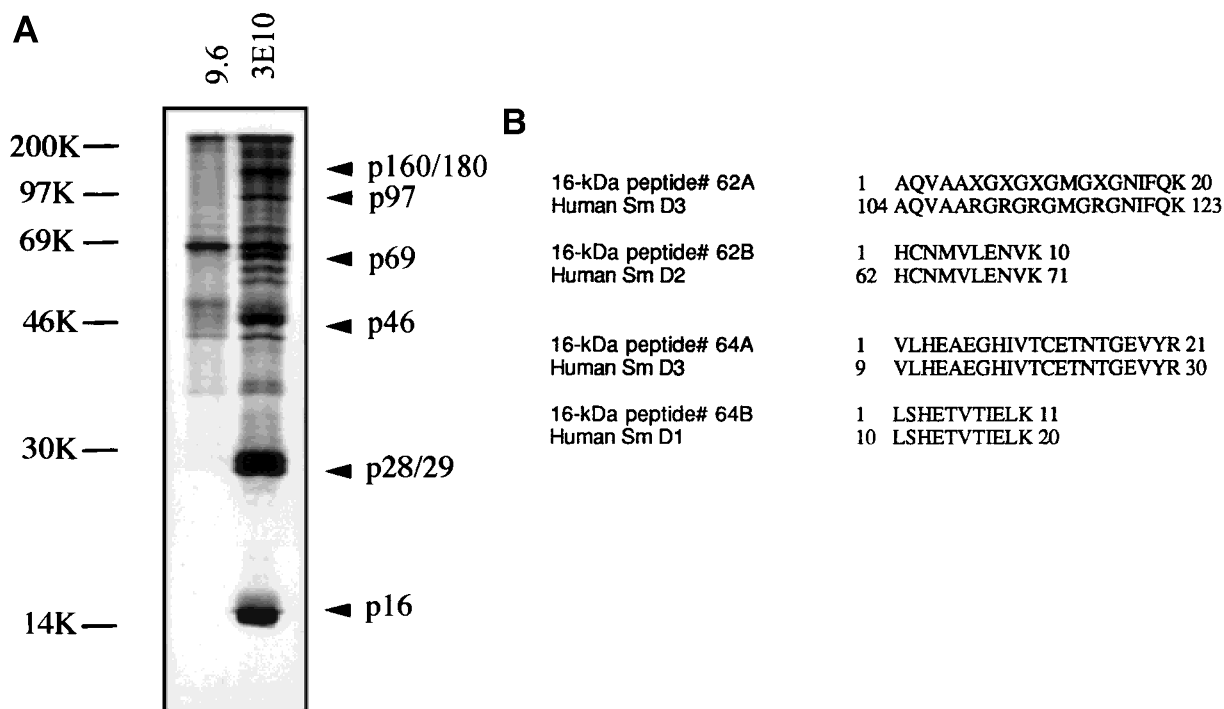

Fig. 1. Monoclonal antibody 3E10 specifically immunoprecipitates spliceosomal Sm proteins. **A:** Immunoprecipitation with mAb 3E10 and control antibody 9.6 (anti-CD2) from [^{35}S]methionine-labeled J32 total cell extract. Proteins in immunoprecipitates were analyzed by 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and protein bands were visualized by fluorography. Apparent molecular weights are indicated on the left side of the gel box. Arrows, major specific bands detected after fluorography. **B:** Alignment of amino acid residues of p16 peptide sequences with corresponding region of homology in the Sm D1, D2, and D3 proteins. Cycles 6, 8, 10, and 14 in this sequence indicated that the same noncommon amino acid derivative at all four positions. We were unable to identify this derivative after consulting several database sources. Mass spectroscopy analysis indicated that the mass of this derivative is 184 Da.

high quantity in the cytosolic fraction of J32 cell extracts. Large-scale immunopurification was performed using soluble protein lysate in a single-step affinity-antibody column made of protein G-Sepharose coated with 3E10 antibody. Eluted proteins were separated by SDS-PAGE and were then electroblotted on a high-retention PVDF membrane. We first focused our attention on the 28/29-kD protein band doublets and the 16 kD band because these proteins were the most prominent proteins in the 3E10 immunoprecipitate. The p16 band was selected first for amino acid sequencing because it separated well from contaminating protein bands, which migrate closely to the 28/29-kD protein doublets. Amido black staining of the proteins on the membrane indicated that approximately 8 μg of p16 protein had been transferred to the filter. The 16-kD band was eluted from the membrane, cleaved, and the cleaved peptides were subjected to reverse-phase HPLC. The best-resolved peptide peaks (peptide 62A, 62B, 64A, and 64B) were analyzed by microsequencing, and peptide sequencing were determined for four different peptides.

BLAST search for amino acid sequence homology results are shown in Figure 1B. We found that peptide 62A and 64A have a near-perfect match with two sequences found in the human Sm D3 protein, while peptides 62B and 64B matched perfectly with sequences found in Sm D2 and Sm D1, respectively [Lührmann et al., 1990]. This finding was quite unexpected because the Sm D protein has no sequence similarity with the peptide sequence used to raise the 3E10 antibody. One of the most prominent feature of the amino acid sequence of D3 protein is the presence of several RG dipeptides (positions 112–119), where arginine is often modified by post-translational modification including dimethylation, respectively [Lührmann et al., 1990] (R. Lührmann, personal communication). It is noteworthy that these five arginine positions were substituted by the same uniden-

tified amino acid derivative in peptide 62A (Fig. 1B).

mAb 3E10 Reacts With a Conformational Epitope of Sm B/B′ Proteins

A careful analysis of three sequences of SmD proteins, D1, D2, and D3) revealed that none of these proteins have the PXXPP(G/X)XR motif. Although Sm D proteins do not have the putative binding epitope for mAb 3E10, prominent Sm D proteins were consistently found in 3E10 immunoprecipitates. We therefore reasoned that mAb 3E10 could bind to other protein(s) having the 3E10 motif and interacting with Sm D or other spliceosomal snRNP proteins. In order to investigate this possibility, we performed immunoprecipitations using 3E10 and anti-Sm (Y12) monoclonal antibodies from ^{35}S-labeled J32 cell lysates, and the immunoprecipitates were then analyzed by SDS-PAGE. The Y12 antibody that we used in this study as a control is not specific to one particular Sm protein, but rather recognizes all the individual Sm protein assembly intermediates [Lehmeier et al., 1994; Lerner et al., 1981]. As shown in Figure 2A, similar patterns were obtained with 3E10 and Y12 antibodies. Several proteins are specifically immunoprecipitated with these antibodies. Among these, we identified the prominent protein doublet 28/29 kD, which is the major band seen with either mAb 3E10 or Y12. The migration of the 28/29-kD doublet corresponded precisely to that of Sm B/B′ protein bands. The intensity of the 28/29-kD band with 3E10 is very comparable with that observed with Y12. Furthermore, the presence of this band in the 3E10 immunoprecipitate was resistant to high strength washing buffers (600 mM NaCl). Taken together, these experiments strongly suggested that 3E10 reacted directly with Sm B/B′, resulting in the

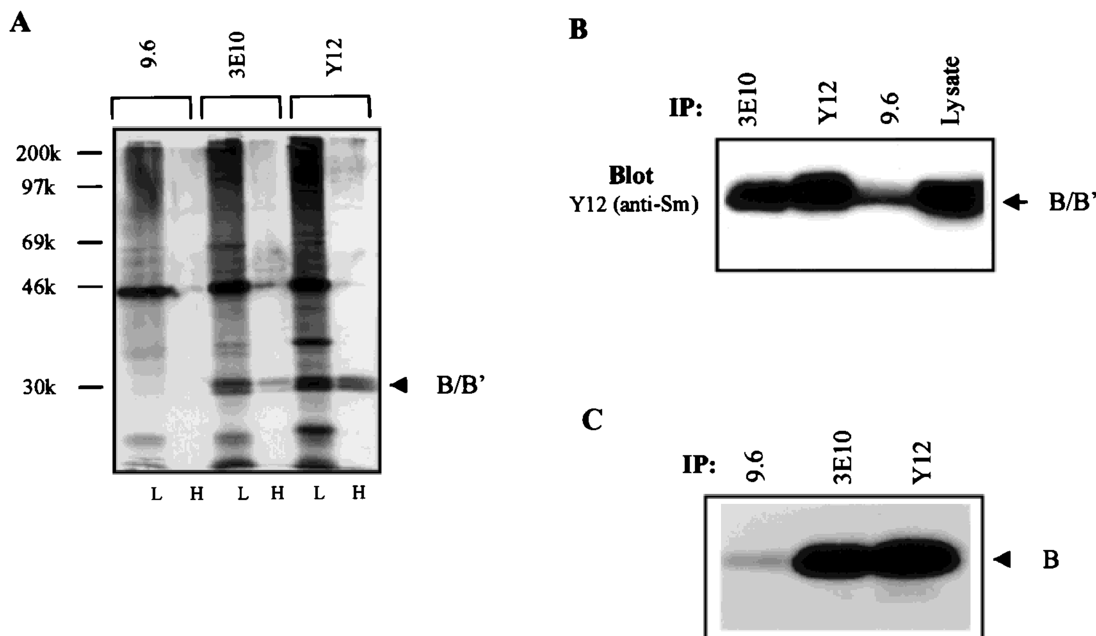

Fig. 2. Monoclonal antibody 3E10 directly bind Sm B/B′ proteins. **A:** Immunoprecipitation with mAb 3E10 and Y12 (anti-Sm) from [^{35}S]methionine-labeled J32 total cell extract. Immunoprecipitates of mAb 3E10 (**lanes 3, 4**) and Y12 (**lanes 5, 6**) from [^{35}S]methionine-labeled extract were washed four times either with a low salt buffer (L) or a high salt buffer (H) containing 0.6 M NaCl, as described under Materials and Methods. Proteins in immunoprecipitates were then eluted by boiling in 2× Laemmli SDS buffer and analyzed by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and protein bands were visualized by fluorography. The SmB/B′ doublet is shown on the right side of the box. **B:** Immunoprecipitates from total J32 extract (20 × 10^{6}) with mAbs 3E10 (**lane 1**), anti-Sm (Y12) antibody (**lane 2**), or the control antibody 9.6 (**lane 3**) were subjected to SDS-PAGE and transferred onto a PVDF membrane. The blot was then probed with anti-Sm B/B′ (Y12) as described under Materials and Methods. A total of 20 μg of proteins from total J32 extract were run in parallel as a positive control (**lane 4**). **C:** In vitro-translated ^{35}S-labeled Sm B protein was immunoprecipitated with 9.6 as a negative control (**lane 1**) 3E10 antibody (**lane 2**), and anti-Sm (Y12) as a positive control (**lane 3**). Immunoprecipitated proteins were eluted by boiling in SDS-PAGE sample buffer and analyzed by SDS-PAGE, followed by fluorography.

co-precipitation of a heteromeric protein complex containing the Sm B/B′ and D proteins. In an attempt to confirm this finding, mAb 3E10 was used to immunoprecipitate the 28/29-kD protein form total J32 cell lysate; the immunoprecipitate was then resolved by SDS-PAGE and immunoblotted with mAb Y12. As shown in Figure 2B, Y12 readily detects Sm B/B′ proteins in the 3E10 immunoprecipitate (lanes 1) but not with the anti-CD2 antibody mAb 9.6 (lane 3); the band observed in the control lane (9.6) represents reactivity with the immunoglobulin light chain. To ascertain the direct binding of 3E10 with SmB protein, we prepared in vitro translated ^{35}S-labeled Sm B protein. As seen by fluorography in Figure 2C, both anti-Sm (Y12) and 3E10 antibodies precipitated in similar amounts the in vitro translated Sm B proteins, whereas there was little Sm B proteins precipitated by the control antibody (9.6). These results indicated that 3E10 binds to the Sm B/B′ proteins.

To further characterize the proteins immunoprecipitated by mAb 3E10, we performed 2D-PAGE experiments. We used NEPHGE to better resolve basic proteins like the Sm proteins. For direct comparison, 3E10 and anti-Sm (SLE serum) immunoprecipitates from J32 cell extract was analyzed in parallel, Figure 3 shows the major proteins found in these two immunoprecipitates, but not in the immunoprecipitate of anti-CD2 mAb 9.6 (not shown). This analysis showed that some proteins displayed a similar pattern in these immunoprecipitates, these proteins include Sm B (28 kD) and Sm D (16 kD), as well as a small but detectable amounts of E (12 kD), F (11 kD), and G (9 kD). The co-immunoprecipitation of E, F, and G was reproducibly less efficient in the immunoprecipitate of 3E10, as compared with that of anti-Sm. The calculated pI value for the Sm B was close to the estimated value from our pH gradient 8.9. We have also observed in several experiments that the 28-kD complex identified above as Sm B displayed in the 3E10 immunoprecipitates a pattern that was slightly distinct from the anti-Sm antibodies used in these experiments. This may represent difference in post-translational modifications of Sm B proteins that are precipitated by these two different antibodies. In addition, it is noteworthy that the U1 snRNP-specific A and C proteins were immunoprecipitated with anti-Sm antibodies, but not with 3E10.

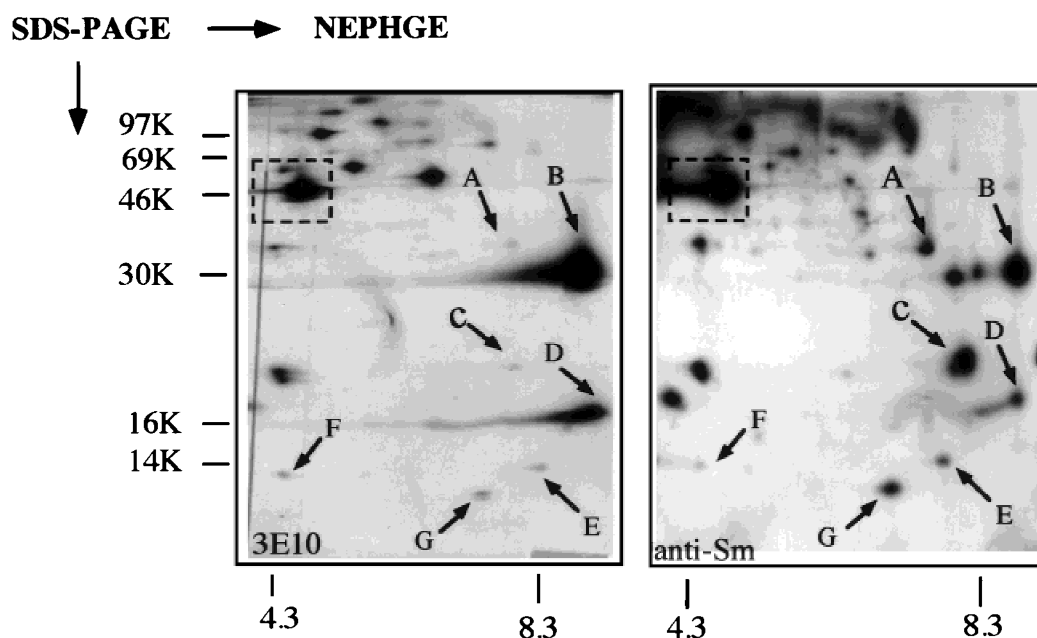

Fig. 3. Two-dimensional nonequilibrium pH gradient gel electrophoresis (NEPHGE) analysis of 3E10-immunoprecipitated complex (left) and anti-Sm-immunoprecipitated complex containing the core Sm proteins and some U snRNP-specific proteins immunoprecipitated with anti-Sm antibodies (right). 25×10^6 of J32 cells were labeled for 4 h with [^{35}S]methionine and total cell extract and used for immunoprecipitation with either 3E10 (left), or with anti-Sm antibodies (serum from an SLE patient, right) as described under Materials and Methods. Dashed boxes, background proteins that are also seen in control 9.6 immunoprecipitations (data not shown).

Sm proteins in the 3E10 Complex Are Not Associated With snRNAs

Because each spliceosomal snRNP consists of one (U1, U2, and U5) or two (U4/6) snRNAs, the Sm core proteins and the snRNP-specific proteins, we examined the reactivity of 3E10 with the U1snRNP-enriched fraction, purified from the nucleus. As shown in Figure 4, the amounts of SmB/B′ and D proteins in the 3E10 immunoprecipitate is considerably less than that seen with the Y12 mAb. Moreover, very little amounts of the U1 A- and C-specific proteins are seen in the 3E10 immunoprecipitate. Furthermore, we examined the snRNA composition in the 3E10 immunoprecipitate complex. Jurkat total cell extract was immunoprecipitated with 3E10, anti-Sm (Y12), or SLE patient serum (anti-Sm), and immunoprecipitates were labeled with T4 RNA ligase using [^{32}P]-pCp nucleotide and subsequently analyzed in parallel on a 10% Sequagel. As shown in Figure 5, anti-Sm but not 3E10 antibody precipitated spliceosomal snRNAs U1, U2, U4/U6, and U5. This result suggests that 3E10 binds preferentially to snRNA-free pool of Sm B/B′ proteins. The weak reactivity of 3E10 with Sm proteins that are associated with U snRNAs and the lack of U1 A and C proteins in the 3E10 immunoprecipitate strongly suggest that 3E10 recognize a subcore of Sm protein complex.

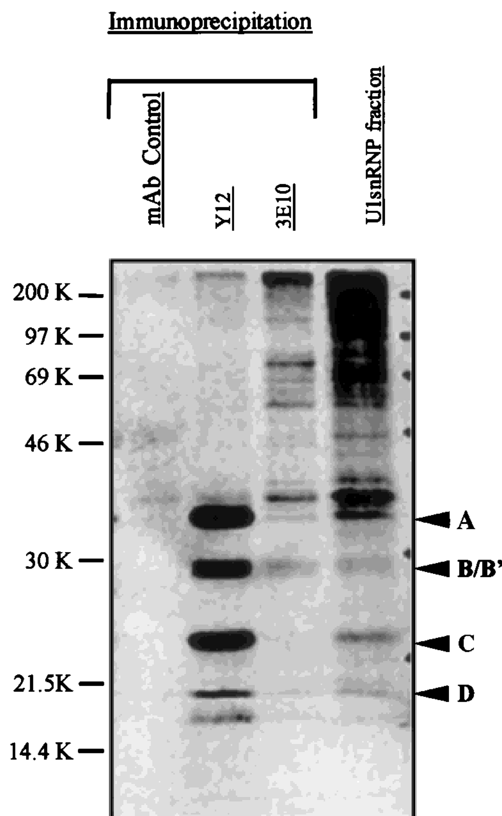

Fig. 4. Monoclonal antibody 3E10 does not immunoprecipitate spilceosomal Sm B/B′ and D proteins from purified nuclear U1 snRNP. U1 snRNP fraction prepared as described under Materials and Methods, was immunoprecipitated with mAb 3E10, Y12, or control antibody. Proteins in the U1 snRNP fraction used for immunoprecipitation were run as a control. Protein samples were analyzed by 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and specific protein bands were visualized by fluorography.

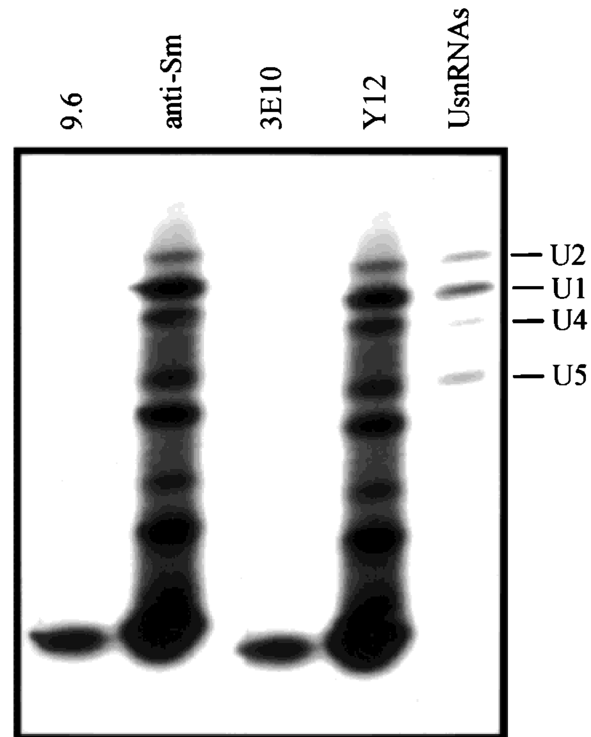

Fig. 5. Monoclonal antibody 3E10 immunoprecipitates snRNA-free spilceosomal Sm proteins. snRNA complexes were immunoprecipitated from J32 total cell extract with anti-Sm antibody Y12 (**lane 4**), anti-Sm antibodies (SLE serum, **lane 2**), 3E10 antibody (**lane 3**), or anti-CD2 antibody 9.6 used as a negative control (**lane 1**). immunoprecipitates were washed with 70% ethanol, resuspended in H_2O, and labeled with the [^{32}P]pCp (0.1 µCi/ml) nucleotide using the T4 RNA-ligase, overnight on ice, the snRNA were re-extracted with the phenol, precipitated with ethanol, and resuspended in 20 µl of the loading buffer. The RNA from each sample was applied on 10% Sequagel containing 8% urea. Purified U snRNA were run in parallel to determine the class of snRNAs in each immunoprecipitate.

DISCUSSION

In this study, we raised a mAb (3E10) specific to a subclass of the polyproline/arg sequence (PPPPPGHR) that was found to form a struc-

tural motif in the CD2 molecule. Interestingly, similar proline sequences (PPPPG(M/I)R) exist in spliceosomal SmB/B′. Because the polyproline-arg sequence in Sm B/B′ is closely similar to the binding motif of 3E10, we used 3E10 as a probe to examine the binding of this antibody to the Sm protein complex and to analyze the protein composition of Sm protein complexe(s) recognized by this antibody. The direct binding of 3E10 to Sm B proteins was confirmed by immunoprecipitations of in vitro-translated ^{35}S-labeled Sm B proteins (Fig. 2C). In addition, a direct comparison of Sm protein complexes in 3E10 and anti-Sm (SLE serum) immunoprecipitates were analyzed in parallel using two-dimensional NEPHGE. The 3E10 complex contained predominantly Sm B and D proteins, and only a small amount of E, F, and G, as compared with the anti-Sm immunoprecipitate (Fig. 3). The presence of D proteins in the 3E10 immunoprecipitate was confirmed by immunoblotting with mAb Y12 (data not shown) and by immunoaffinity protein purification and peptide microsenquencing of peptide digest of the 16-kD protein band, which showed several sequences that matched the sequence found in Sm D1, D2, and D3 proteins (Fig. 1B). Although the exact stoichiometry of the B/B′ and D proteins cannot be determined here, the analysis of proteins by SDS-PAGE showed roughly equivalent Amido Black staining intensity for B and D proteins (not shown). Another consistent finding in the 3E10 complex is the lack of the U1 snRNP-specific A and C proteins.

The sequence PPPPG(M/I)R found in Sm B/B′ appears very similar to the binding motif of mAb 3E10, which was investigated in a separate study using an M13-displayed random peptide library. mAb 3E10 binds preferentially to the sequence motif PXXPP(G/X)XR. The flanking proline residues and their exact positioning, as well as the presence of Arg at the C-terminus, are necessary for optimal mAb 3E10 binding mAb 3G12, another antibody that was raised against the polyproline-rich sequence (ATSQHPPPPPGHRSQ), failed to bind the PXXPP(G/X)XR sequence motif and SmB/B′ proteins (data not shown). The absence of snRNP C or snRNP A in the 3E10 immunoprecipitate strongly indicates that the antigenic region spanning amino acids 117–126 PAPGMRPP in the snRNP C and the PPPGMIPP sequence in snRNP A [James et al., 1992, and references therein] are not recognized by 3E10. This can be expected because the additional flanking proline residues and their exact positioning, as well as the presence of Arg at the C-terminus, are necessary for optimal mAb 3E10 binding. The question of whether other components in the snRNP protein core also have the polyproline-arg motif seen with 3E10 remains to be investigated. Immunoblotting experiments showed that mAb 3E10 reacts with several protein bands, which include the CD2 protein and two other unidentified proteins with an apparent molecular weight of 80 kD and 160 kD (data not shown).

In addition to the common snRNP proteins Sm B′, B, D, E, F, G, several other proteins are found in 3E10 immunoprecipitates were further analyzed by a direct comparison of protein patterns in the 3E10 and anti-Sm immunoprecipitates using 2D-PAGE. These proteins migrated with an apparent molecular weight of ≥46 kD (data not shown). It is difficult to assess whether any of these proteins corresponds to previously identified snRNP-specific proteins or other non-snRNP factors. Immunoblotting of the 3E10 immunoprecipitate with anti-U1 70K antibodies showed the presence of the U1 70K protein in the 3E10 complex (data not shown). However, this observation is difficult to reconcile with the absence of the U1 A and C proteins in the 3E10 complex (Fig. 4). Solely on the basis of 2D-PAGE, it is not possible to provide more than a tentative estimate of the real number of these proteins in 3E10 complex. This will be clarified only through purification of the complex and protein sequence analysis, which will permit better characterization of these proteins and identification of any structural relationship between them. Despite this, it is clear that the immunoprecipitate of 3E10 contains more than the Sm proteins.

Interestingly, immunoprecipitation experiments showed that the Sm protein in the 3E10 complex are not associated with any of U1, U2,U4/U6, or U5 snRNAs. However, as expected, UsnRNA was co-precipitated with Y12 as well as anti-Sm (Fig. 5). It was previously shown that both Y12 and anti-Sm precipitated similar amounts of U snRNA from the cytoplasm and the nucleus of Hela cell extracts [Mattaj et al., 1986; Fisher et al., 1990, 1993]. Taken together, our data strongly suggest that 3E10 binds preferentially to snRNA-free pool of Sm proteins and that these polypeptides are present in stoichiometric amounts within a

RNA-free, Sm protein complex. The latest finding raises important questions about the exact protein composition of these complexes and their specific presence as a functional intermediate in the assembly of the snRNP core particle. This observation can be investigated by co-immunoprecipitations with 3E10 antibody of in vitro-translated [^{35}S]methionine-labeled premixed Sm proteins, as well as co-immunoprecipitation assays of reconstituted snRNP particles in vitro with various subsets of the core proteins.

The fact that there is no [^{32}P]-pCp-labeled snRNA in the 3E10 immunoprecipitate indicates that the 3E10 complex is very unlikely to be an assembled snRNP. It is our preliminary judgment that 3E10 preferentially recognize an snRNA-free B/B′-D3 protein complex. However, it remains unclear why the 3E10 immunoprecipitate contains D1, D2, E, F, and G proteins. A likely explanation is that weak protein-protein interactions may exist between D1/D2 and EFG complexes. The in vivo evidence of one or more heteromeric complex(es) containing Sm B/B′, D, E, F, and G proteins suggested by our studies is consistent with previous studies [Sauterer et al., 1990; Feeney et al., 1989; Raker et al., 1996]. Pulse-chase gradient fractionation of the cytoplasmic proteins labeled in vivo with [^{35}S]methionine and precipitations with Y12 antibody have indicated that E, F, G, and one or more D proteins co-sedimentated at 6S [Fisher et al., 1985; Sauterer et al., 1990]. Thus, it was suggested that these proteins form an RNA-free heteromeric complex that bind as such to the snRNA Sm site followed by the addition of B/B′ and D3 proteins. More recently [Raker et al., 1996], studies of in vitro formation of RNA-free core proteins and of UsnRNA have provided evidence for an interaction between B or B′ and the D3 protein. Sm B/B′ and D3 can form a stable complex in the absence of U1RNA, this complex is extremely stable withstanding buffers containing up to 750 mM KCl [Raker et al., 1996]. It also indicated that there is an ordered snRNP core assembly pathway that involves the initial formation of D1-D2, E-F-G, D1-D2-E-F-G, and B/B′-D3 complexes, the subsequent association of U snRNA with D1-D2-E-F-G, to form an snRNP subcore particle, and finally, the recruitment of the trimeric protein complex made of B/B′-D3 complex to generate an snRNP core particle functional in 5′ cap hypermethylation [Raker et al., 1996].

The finding that mAb recognizes an RNA-free Sm protein complex but not Sm B proteins that are assembled with the UsnRNA, suggests that the epitope binding for mAb may become unaccessible upon heterooligomerization of Sm B-D3 and subsequent interactions with other Sm protein complexes and with U snRNA. Functional important intra- and/or intermolecular structural domains are thought to be exposed or created upon heterooligomerization and are necessary for subsequent interactions with other Sm protein complexes and with U snRNA as well [Raker et al., 1996]. Consistent with this hypothesis is the observation that 3E10 failed to react with intracellular proteins in indirect immunofluorescence assay using Hep-2 permeabilized-fixed cells, suggesting that the proline-rich epitope is inaccessible in vivo.

The role of the repeated proline-rich motif in Sm B/B′ proteins is unkown. It is conceivable that the Sm proline-rich motif represents a possible consensus binding motif for undefined ligands. This may provide Sm B proteins with more than one region for contact with an interacting protein domain and may provide another level of specificity derived from these multiple interactions. Prior interactions of several Sm proteins appear to be a prerequisite for RNA binding [Raker et al., 1996]. The identification of numerous protein-protein interactions among the Sm proteins suggests that several of these proteins contains multiple interaction domains. Thus, PPPPG(M/I)R sequences in Sm B/B′ can potentially form interaction domains created upon heterooligomerization and necessary for the assembly of snRNP particles. This can be investigated by inhibition studies with 3E10 of reconstituted snRNP particles in vitro with various subsets of the core proteins. Furthermore, deletion of one of multiple proline-rich motifs in the Sm B proteins should help clarify whether this repeated motif is involved in the formation of the final snRNP particle.

Alternatively, the close similarity of Sm B/B′ proline rich-repeats to SH3 class II ligands [Yu et al., 1992, 1994; Feng et al., 1994; Alexandropoulos et al., 1995; Knudsen et al., 1995; Weng et al., 1995; Rickles et al., 1995] may suggest a similar function. Interactions mediated by Sm B/B′ proline-rich sequences PPPPGM/IR may be important for targeting of specific snRNP core proteins to specific subcellular compartment or for connecting receptor engagement at

the plasma membrane with regulatory events involving the snRNP core assembly pathway.

To our knowledge, 3E10 is the first well-characterized mAb specific for a subclass of polyproline-arg motif recognizing Sm B/B′ and CD2 proteins. 3E10 antibody can be used to further characterize the nature of protein components in the snRNA-free Sm subcore protein complex(es) that are formed during the snRNP core assembly steps. A comprehensive analysis with the 3E10 antibody of the protein composition of Sm protein complex intermediates will allow us to to study the potential role of Sm B/B′ proteins proline-rich motif in protein interactions that may be necessary for the proper snRNP core assembly.

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