Promiscuous Modification of the Nuclear Poly(A)-binding Protein by Multiple Protein-arginine Methyltransferases Does Not Affect the Aggregation Behavior*

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The mammalian nuclear poly(A)-binding protein, PABPN1, carries 13 asymmetrically dimethylated arginine residues in its C-terminal domain. By fractionation of cell extracts, we found that protein-arginine methyltransferases (PRMTs)-1, -3, and -6 are responsible for the modification of PABPN1. Recombinant PRMT1, -3, and -6 also methylated PABPN1. Our data suggest that these enzymes act on their own, and additional polypeptides are not involved in recognizing PABPN1 as a substrate. PRMT1 is the predominant methyltransferase acting on PABPN1. Nevertheless, PABPN1 was almost fully methylated in a Prmt1−/− cell line; thus, PRMT3 and -6 suffice for methylation. In contrast to PABPN1, the heterogeneous nuclear ribonucleoprotein (hnRNPK) is selectively methylated only by PRMT1. Efficient methylation of synthetic peptides derived from PABPN1 or hnRNPK suggested that PRMT1, -3, and -6 recognize their substrates by interacting with local amino acid sequences and not with additional domains of the substrates. However, the use of fusion proteins suggested that the inability of PRMT3 and -6 to modify hnRNPK is because of structural masking of the methyl-accepting amino acid sequences by neighboring domains. Mutations leading to intracellular aggregation of PABPN1 cause the disease oculopharyngeal muscular dystrophy. The C-terminal domain containing the methylated arginine residues is known to promote PABPN1 self-association, and arginine methylation has been reported to inhibit self-association of an orthologous protein. Thus, arginine methylation might be relevant for oculopharyngeal muscular dystrophy. However, in two different types of assays we have been unable to detect any effect of arginine methylation on the aggregation of bovine PABPN1.

Dimethylation of arginine side chains is a relatively common post-translational modification of proteins (1–5). Most proteins modified in this manner are involved in DNA or RNA function; many of them bind nucleic acids directly, and the modification is frequently found in RNA binding domains. Still, modulation of protein-protein rather than protein-nucleic acids interactions seems to be the predominant function of arginine dimethylation (2, 3). There are two types of arginine dimethylation as follows. Symmetric dimethylation (ω-N\textsubscript{G},N\textsubscript{G}-dimethylarginine) has been found in some of the so-called Sm proteins in the core of spliceosomal small nuclear ribonucleoprotein particles (6) and in several other proteins and is catalyzed by the type II protein-arginine methyltransferases (PRMT)\textsuperscript{2}–5, -7, and -9 in mammalian cells (4). Asymmetric dimethylation (ω-N\textsubscript{G},N\textsubscript{G}-di-methylarginine) is the more frequent modification and is catalyzed by the type I protein-arginine methyltransferases PRMT1, -3, -4, -6, and -8 (4). PRMT2 is considered a type I enzyme because of sequence similarities, but catalytic activity has not been demonstrated. The human genome contains two more potential PRMTs, which have not been characterized (4).

The substrate preferences of different type I enzymes are poorly understood, and in many cases it is not clear whether a particular protein is methylated by a specific PRMT or by multiple enzymes. For some proteins, in vivo methylation by one specific methyltransferase has been confirmed by genetic or quasi-genetic means. For example, a knock-out of Prmt1 abolishes the methylation of the proteins Sam68 (7), hnRNPK (8), and histone H4 (9). The role of PRMT1 in H4 methylation has also been confirmed by RNA interference experiments (10). A hypomorphic allele of Prmt3 leads to a loss of methylation of ribosomal protein S2 in mice (11), and similar results have been obtained with a prmt3 deletion in Schizosaccharomyces pombe (12). A knock-out of Prmt4 abolishes the modification of the cytoplasmic poly(A)-binding protein and the transcriptional cofactor p300 (13), and knockdown of PRMT6 interferes with histone H3 methylation at Arg-2 (14, 15). These data suggest specific enzyme-substrate relationships, and a published survey

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also indicates that PRMT4 and -6 modify unique substrates (3). In contrast, most identified PRMT3 substrates can also be modified by PRMT1 according to the same survey; this argues for redundant PRMT function.

The features of substrate proteins directing their modification by one or more PRMTs also remain to be defined. RGG domains with the consensus sequence (F/G)GGRRG(G/F) (1, 16) were initially identified as prototypical arginine methylation sites. However, many identified substrates do not correspond to this consensus, and a more general definition of a methylation substrate is called a glycine- and arginine-rich sequence. A GST-glycine- and arginine-rich fusion protein (17) and synthetic peptides with similar sequences indeed serve as generic methyl acceptor sites, being methylated, in vitro, by PRMT1, -3, -6 and by the type II enzyme PRMT7 (3) but not by PRMT4 (18, 19). This supports the idea that at least some of the PRMTs may be largely redundant with respect to their substrates.

One unbiased approach to the identification of the PRMT(s) relevant for the modification of a specific substrate is purification from a cell extract. For example, this approach has led to the identification of PRMT1 as being responsible for the methylation of Arg-3 in histone H4 (9). In contrast, experiments based solely on the use of recombinant PRMTs are potentially flawed. Many experiments have used GST fusion proteins, and this type of fusion affects the activity of some PRMTs but not others, skewing their relative efficiencies in the modification of a substrate (8). More importantly, for most PRMTs it is currently unknown whether they act by themselves or are merely the catalytic subunits of larger complexes that may influence the substrate specificity. Locus-specific arginine methylation of histones strongly suggests roles for additional proteins in targeting methylation (2, 20). In fact, the methylation of histone H3 Arg-17 by PRMT4 (=CARM1) depends on the recruitment of the enzyme by a transcriptional coactivator (18). PRMT4 has also been identified as a component of a hetero-oligomeric complex, which can methylate histone H3 within nucleosomes, whereas PRMT4 by itself prefers the free histone as a substrate (21). The type II enzyme PRMT5 forms a complex with two other proteins, WD45 and plCh. The latter is responsible for substrate recognition by binding to the Sm domain (22–24).

The nuclear poly(A)-binding protein (PABPN1) binds the growing poly(A) tails during 3’-processing of mRNA precursors in the cell nucleus (25–28). It stimulates the activity of poly(A) polymerase by increasing the processivity of the enzyme and contributes to limiting the poly(A) length to about 250 nucleotides (27, 29, 30). The protein contains a C-terminal domain of ~49 amino acids, in which all 13 arginine residues are quantitatively asymmetrically dimethylated (31, 32). The methylated sites in mammalian PABPN1 are clusters of RXR sequences, which reside in a sequence environment reminiscent of RGG domains, being rich in phenylalanine and tyrosine and low in sequence complexity. The C-terminal domain contributes both to poly(A) binding and to the stimulation of polyadenylation, but neither function is noticeably influenced by arginine methylation (29, 33). PABPN1 was found to be methylated by recombinant PRMT1 and -3 (32). However, this analysis was incomplete; other PRMTs were unknown at the time and thus not tested, and only recombinant GST-PRMT fusion proteins were used. Thus, the question of which enzymes are responsible for the modification of PABPN1 deserved to be re-evaluated.

Mutations in the human gene encoding PABPN1 cause a genetic disease, oculopharyngeal muscular dystrophy (OPMD). These mutations lead to the extension of the polyalanine tract at the N terminus of the protein from 10 residues in the wild-type up to 17 residues (34). The mutant protein forms regular fibrillar aggregates in muscle cell nuclei of afflicted individuals (35). Wild-type PABPN1 has a tendency to form ill-defined amorphous aggregates in vitro (31, 36, 37). Formation of these PABPN1 aggregates relies to a large extent on the C-terminal domain containing the dimethylated arginine residues (33, 38). It has been proposed that formation of amorphous aggregates may promote formation of fibrils (38). Several observations have indicated that arginine methylation might affect protein aggregation. In immunoprecipitation experiments with cells lacking PRMT1, the background was strongly increased compared with wild-type cells, suggesting a generally increased tendency of proteins to aggregate (8). The Saccharomyces cerevisiae protein Npl3, which normally carries asymmetric dimethylarginine, has an increased tendency to self-associate in a methylation-deficient yeast strain (39). The PABPN1 orthologue in S. pombe (termed Pab2) was recently identified and shown to be arginine-methylated in RG dipeptides in its C-terminal domain. Again, genetic inactivation of the methyltransferase RMT1 increased the tendency of Pab2 to self-associate, and it was proposed that this observation might be relevant for PABPN1 fibril formation in OPMD (40).

In this study, we identify the type I arginine methyltransferases PRMT1, -3, and -6 as the enzymes modifying mammalian PABPN1. Among them, PRMT1 is the dominant enzyme. Our data suggest that PRMT1, -3, and -6 recognize their substrates on their own, independently of associated polypeptides, and that their overlapping but distinct substrate specificities are determined both by local amino acid sequences and by accessibility of methyl-accepting side chains in the context of a folded protein. Comparison of methylated and unmethylated PABPN1 in two different assays revealed no methylation-dependent difference in self-association, whereas the same assays showed that a His tag does favor aggregation.

**Experimental Procedures**

Plasmids—Bovine His-PABPN1 and His-PABPN1ΔC49 were expressed from the plasmids pHK-synPABPN1 and pHK-synPABPN1ΔC49, respectively (33). For expression of non-tagged PABPN1, the Ndel/BamHI fragment of pHK-synPABPN1 containing the PABPN1 open reading frame was cloned into the corresponding sites of pET11a (Novagen). PABPN1-KS and PABPN1-KL are fusion proteins in which the C-terminal domain of PABPN1 was replaced by fragments of hnRNP K containing the methyl-accepting arginines. The fragment encoding amino acids 258–306 in the expression construct for His-tagged bovine PABPN1 was removed by means of an introduced KpnI site and the BamHI site. This fragment was replaced either by a DNA sequence encoding amino acids 241–333 of hnRNP K, amplified with the primers CGGGGTACCT-
Arginine Methylation of the Nuclear Poly(A)-binding Protein

TTGATGACGCTGCG and CGCGGATCTCTAAGACGCTGCCAGGCTCAGTAACCGGTTCCGAGC (PABPN1-KL fusion), or by a DNA sequence encoding amino acids 251–309, amplified with the primers CCGGGATCGCAGTTCCATCGGG and CGCGGATCTTCAAGAGAATTCCGAGGC (PABPN1-NS fusion). The plasmid for recombinant expression of the llama single chain antibody fragment anti-PABPN1 (3F5) was a gift of Peter Verheesen, University of Utrecht (41). The plasmid pET28b-PRMT1 (42) used to express rat His-PRMT1 with a deletion of the first 10 amino acids was a gift of Xiaodong Cheng, Emory University. Human His-PRMT3 was expressed from the plasmid pET28a-PRMT3 (8). The associated human proteins PRMT5, His-pICln, and WD45 were encoded by the plasmid pET28a-pICln-PRMT5-WD45, obtained from Lucy Handoko from a clone obtained from B. Laggerbauer, University of Würzburg, Germany, with the primers 5′-CATGCCATGGAAATTTTGAGCCACC-3′ and 5′-CGCGGATCCTCAGGCATCGGTAGCT-3′. The PCR product was digested with Ncol and BamHI and subcloned into pET19b (Novagen).

Plasmids for expression of nontagged PRMTs were generated as follows. Human Prmt1, splice variant 2 (43) (GenBank™ accession number AF222689), was PCR-amplified from a clone obtained from B. Laggerbauer, University of Würzburg, Germany, with the primers 5′-CATGCGATTGGAATTTTGAGCCACC-3′ and 5′-CGCGGATCCTCAGGCATCGGTAGCT-3′. The PCR product was digested with Ncol and BamHI and cloned into pET19b. These enzymes excised the His tag from the plasmid. Prmt3 was amplified from the His-PRMT3 construct (8) with the primers 5′-CATGCCATGGAAATTTTGAGCCACC-3′ and 5′-CGCGGATCCTCAGGCATCGGTAGCT-3′ and cloned as described for PRMT1. Prmt6 was as amplified from the GST fusion construct (8) with the primers 5′-GGGAATTCCATATGTCGCAGCCCAAG-3′ and 5′-CGCGGATCCTCAGGCATCGGTAGCT-3′. The fragment was digested with Ndel and BamHI and inserted into pIT7-7 (44). All constructs were confirmed by sequencing.

Recombinant Proteins—Expression and purification of His-PABPN1 and His-PABPN1ΔC49 were performed as described in Ref. 33 except that sometimes a Resource S column (GE Healthcare) was used as the second step. Both PABPN1-K fusions were purified by the same procedure with a Mono Q column as the second step. For the purification of nontagged PABPN1 from Escherichia coli, the lysate was applied to a Blue- Sepharose column. Elution and further purification were as described (45). Recombinant hnRNP K was generously provided by Bodo Moritz (University of Halle). Purified recombinant SmD3 and SmB/B′ were a kind gift of Utz Fischer. His-PRMT1, -3, and -6 were expressed and purified as described in Ref. 8. For use in kinetic measurements, His-PRMT1 and -3 were further purified on a Mono Q column (GE Healthcare) and His-PRMT6 on a Mono Q and a phenyl-Superose column. The nontagged PRMTs were purified by slight variations of the following procedure. The bacterial lysate was loaded on a DEAE-Sepharose column (GE Healthcare; 10 mg of protein/ml column volume) equilibrated in 50 mM Tris–HCl, pH 7.4, 50 mM KCl, 1 mM EDTA, 0.5 mM dithiothreitol, and 10% glycerol. The PRMTs were eluted with a KCl gradient from 50 to 500 mM KCl. Fractions active in the methylation assay (see below) were pooled and loaded on a hydroxyapatite column (Bio-Gel HTP, Bio-Rad; up to 5 mg of protein/ml column volume). For PRMT1 and -3, the equilibrium solution contained 10% glycerol, 0.5 mM dithiothreitol, and either 1 mM MgCl₂ (PRMT1) or 1 mM NaCl (PRMT3). Protein was eluted in five steps from 0.01 to 0.3 M potassium phosphate, pH 8. For PRMT6, DEAE peak fractions were dialyzed against 25 mM Hepes, 1 mM KCl, 10% glycerol, 1 mM dithiothreitol, and 0.01% Nonidet P-40, pH 7.5, and loaded onto a hydroxyapatite column. Following a washing step with 25 mM Hepes, 1 mM potassium phosphate, 10% glycerol, 1 mM dithiothreitol, and 0.01% Nonidet P-40, pH 7.5, the protein was eluted with a linear potassium phosphate gradient. Peak fractions from all hydroxyapatite columns were concentrated with a Centriprep device (Millipore), and 200 μl were loaded on a Superdex 200HR 10/30 column (GE Healthcare) run in 20 mM Tris–HCl, pH 7.4, 100 mM KCl, and 10 mM dithiothreitol (for PRMT1 and -3) or 25 mM Hepes, pH 8.0, 100 mM KCl, 10% glycerol, 1 mM dithiothreitol, for PRMT6. For gel filtrations, fractions containing PRMT1 or -3, 10% glycerol was added for storage.

Concentrations of PRMT1 and -6 and all methylation substrates were determined by photometric measurements based on calculated extinction coefficients. Concentrations of PRMT3 were determined by SDS-PAGE, subsequent Coomassie staining, and comparison with known amounts of bovine serum albumin.

Synthetic Peptides—Peptides were synthesized with a free N terminus and an amidated C terminus by N-(9-fluorenyl)methoxycarbonyl/tert-butyl strategy and solid-phase peptide synthesis with the help of a peptide robot system (Syro; MultiSynTech, Witten, Germany) as described (46). After cleavage from the resin, the peptides were purified by preparative HPLC and analyzed by reversed-phase analytical HPLC. Identity of the peptides was confirmed by MALDI-TOF mass spectrometry (Ultraflex III; Bruker Daltonic).

Antibodies and Immunological Procedures—Anti-PRMT1 was generated against GST-PRMT1 by Eurogentec. An anti-PRMT1 serum obtained from Stephane Richard, McGill University, was also used (7). Antibodies directed against PRMT2 and -3 were described in Ref. 8. Anti-PRMT4 was purchased from Upstate (catalog number 07-080). Anti-PRMT5, anti-WD45, and anti-pICln were kind gifts of Utz Fischer, University of Würzburg. Anti-PRMT6 was purchased from Imgenex (catalog number IMG-506). Rabbit serum raised against bovine PABPN1 has been published previously (47). Single chain llama antibody directed against PABPN1 (3F5) was expressed and purified as described (41).

Anti-PRMT1 and anti-PRMT3 were affinity-purified. The respective His-tagged antigens were immobilized on CNBr-activated Sepharose beads (GE Healthcare). Residual activated groups were blocked with 10 mM Tris–HCl, pH 8.0. The beads were washed with 10 volumes of 1× PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO₄, 2 mM KH₂PO₄, pH 7.4), 1 volume of 100 mM glycine, pH 2.7, and three times with 10 volumes of 1× PBS. Antiserum was diluted with 1 volume of 10 mM Tris–HCl, pH 7.4, 2 mM EDTA, 4 mM EGTA, 10 μg/ml leupeptin, 5 μg/ml pepstatin, 5 μM PMSF, added to the beads, and incubated for 2 h at room temperature. The beads were washed three times with
Arginine Methylation of the Nuclear Poly(A)-binding Protein

10 volumes of 1× PBS, and antibody was eluted with 100 mM glycine, pH 2.7, and immediately neutralized by the addition of 100 mM Tris-HCl, pH 8.0 (final concentration).

For Western blot analysis, proteins were loaded on a 10 or 12% SDS-polyacrylamide gel and blotted to a polyvinylidene difluoride (Millipore) or nitrocellulose membrane overnight in 192 mM glycine, 2.7 mM Tris, containing 20% methanol in the case of nitrocellulose. Membranes were blocked with 2–5% low fat milk in TBST (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.05% Tween 20) or PBST (137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 2 mM KH2PO4, 0.05% Tween 20). Primary antibodies were diluted in the same buffers. Secondary antibody (peroxidase-linked anti-rabbit or anti-mouse; GE Healthcare) was diluted 1:5000 or 1:2000 in 2–5% low fat milk in PBST. The Myc-tagged llama antibody was detected by an anti-Myc antibody (Invitrogen) diluted 1:2000 in PBST prior to incubation with anti-mouse peroxidase-linked antibody. Blots were developed with West Pico Luminol/Enhancer solution (Pierce).

Purification of PRMT1 from Cytoplasmic HeLa Extract—All steps were performed in the cold. Extract or pooled fractions were dialyzed against the column equilibration buffer and centrifuged at 10,000 × g if not indicated otherwise. All fractions were assayed for their methylation activity as described below. The distributions of PRMT1, -2, -3, -4, and -6 were analyzed by Western blot assays.

DEAE-Sepharose chromatography of HeLa cell extract was the column described in Ref. 8. Methylation activity directed against PABPN1 was found in two overlapping peaks. Fractions corresponding to the first peak, containing PRMT1 and -3, were pooled and loaded on a Macroprep-S column (7 mg of protein per ml column volume) equilibrated in buffer 1 (50 mM Hepes, pH 7.4, 50 mM KCl, 1 mM EDTA, 0.5 mM dithiothreitol, 10% glycerol). The column was washed with 1 volume of buffer 1. Both PRMTs were recovered in the flow-through and wash fractions. They were pooled and adjusted to the conductivity of buffer 2 (20 mM Tris-HCl, pH 7.4, 500 mM ammonium sulfate, 1 mM EDTA, 0.5 mM dithiothreitol, and 10% glycerol) by addition of ammonium sulfate. Precipitates were removed by centrifugation, and the supernatant was applied to a phenyl-Sepharose column (8 mg of protein per ml column volume) equilibrated in buffer 2. Proteins were eluted with a gradient from 500 to 0 mM ammonium sulfate. The methylation activity eluted from 150 to 200 mM ammonium sulfate and could be divided into two activity peaks as follows: the first contained mainly PRMT1 and the second PRMT1 and PRMT3. Fractions containing mainly PRMT1 were concentrated by centrifugation through an Amicon filter and loaded on a Superdex 200 Hiload 16/60 gel filtration column (8 mg of protein per ml column volume) equilibrated in 25 mM Tris-HCl, pH 7.4, 1% Triton 100, 1 mM dithiothreitol, 10 μg/ml leupeptin, 5 μg/ml pepstatin, 5 μM PMSF. Lysate was centrifuged for 30 min at 20,800 × g, and the supernatant was dialyzed and loaded on a DEAE-Sepharose Fast Flow column (GE Healthcare) (10 mg of protein per ml column volume) equilibrated in buffer A containing 50 mM KCl. The column was washed with 1 volume of the same buffer, and proteins were eluted by a gradient from 50 to 500 mM KCl in buffer A. The methylation activity found in the fractions of the wash step and subsequent first fractions coeluted with PRMT3 and PRMT4. These fractions were pooled (pool Ila), and 25% was loaded on a 1-ml Mono S column, equilibrated in buffer B containing 50 mM KCl. Proteins were eluted by a gradient from 50 to 500 mM KCl in buffer B. The main methylation activity of the DEAE-Sepharose eluted from 150 to 250 mM KCl (pool Iib) and contained PRMT5 and -6. One representative column fraction of pool Iib was concentrated by centrifugation through an Amicon filter and loaded on a Superdex 200 HR 10/30 equilibrated in buffer A containing 50 mM KCl. This column separated the methylation activity into two pools as follows: pool IIa contained PRMT5, and pool IIb contained PRMT6 and a negligible amount of PRMT5. Both pools were separately loaded on a 1-ml Mono Q column equilibrated in buffer C containing 50 mM KCl. The column was washed with 2 volumes of buffer C, and proteins were eluted by a gradient from 50 to 500 mM KCl in buffer C.

Purification of PABPN from Tissue—Purification of calf thymus was according to the published procedure (45). For purification from Prmt1−/− ES cells, the same protocol was used. Fractions of the DEAE-Sepharose fast flow column (see above) containing PABPN1 were identified by Western blotting and used for further purification to homogeneity.

In Vitro Methylation Assay—Methytransferase activity was assayed in a reaction volume of 25 μl containing 50 mM Hepes, pH 8.0, 40 mM potassium acetate, 0.2 mg/ml bovine serum albumin, 0.01% Nonidet P-40, 10% glycerol, 1 mM EDTA, 0.5 mM dithiothreitol, 40 μM S-adenosyl-L-[methyl-14C]methionine (GE Healthcare), and 500 ng of PABPN1 (~0.6 μM). The reaction was incubated at 30 °C for the time indicated. The incorporation of methyl groups was measured by SDS-PAGE, phosphorimaging, and quantification by the ImageQuant software (GE Healthcare). A standard curve with known quantities of labeled S-adenosylmethionine was generated for each experiment and used to calculate molar quantities of methyl group incorporation. One unit was defined as 1 pmol of methyl groups incorporated in 3 h.

For steady-state kinetics, reactions were performed in a total volume of 20 μl under standard conditions. S-Adenosylmethionine was titrated up to 85 μM with PABPN1 held constant at 0.9 μM. PABPN1 and hnRNP K were titrated up to 1.8 or 1.7 μM, respectively, with S-adenosylmethionine held constant at 42 μM. Peptides were titrated up to a concentration of 10× Km or higher except the K1 peptide in the PRMT1 reaction and the K2 peptide in the PRMT3 reaction, which were titrated up to 4× Km. A linear dependence of the reaction velocity on enzyme concentration was experimentally verified, and reaction times were chosen to reflect initial rates. Typically, each data point was done in duplicate or triplicate, and each titration was carried out at least twice. Km and Vmax values were calculated.
either directly from hyperbolic fits or from linear fits to the Lineeweaver-Burk equation. \( k_{\text{cat}} \) was calculated from \( V_{\text{max}} \). In view of the highly probable ordered sequential mechanism (48), all kinetic constants given should be considered apparent constants.

**Cell Culture**—Culture of ES cells (obtained from Earl Ruley, Vanderbilt University) and metabolic labeling with \( \text{L-}[^{3}H] \text{methionine} \) (70–85 Ci/mmol; GE Healthcare) in the presence of translation inhibitors were as described (8). HeLa cells were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen) and 1% penicillin/streptomycin (Invitrogen). For the quantitative analysis of PRMT content by Western blotting, cells were trypsinized and lysed in 50 mM Tris-HCl, pH 7.4, 300 mM NaCl, 1% SDS, 1 mM dithiothreitol, 10 \( \mu \)g/ml leupeptin, 5 \( \mu \)g/ml pepstatin, 5 \( \mu \)M PMSF. DNA was digested with DNase I (Roche Applied Science) prior to centrifugation for 30 min at 20,800 \( \times \) g.

**Mass Spectrometric Analysis**—After desalting with ZipTip C4 (Millipore), protein masses of PABPN1 purified from PRMT1–3 ES cells were determined with a Q-TOF 2 mass spectrometer (Waters) equipped with a modified nano-ESI source to hold a pico-tip (New Objective, Cambridge, MA). The samples were injected with a syringe pump (Harvard Apparatus, MA) with a flow rate of 300 nl/min. The MaxEnt\textsuperscript{TM} algorithm was used for deconvoluting the data for single charge state. Mass accuracy was \( \pm 2 \) Da.

For the analysis of \textit{in vitro} methylation, 500 ng of His-PABPN1 was methylated by either 1 \( \mu \)g of His-PRMT1 (3 h of incubation), 2 \( \mu \)g of His-PRMT3 (1 h of incubation with 1 \( \mu \)g followed by 1 h with an additional 1 \( \mu \)g), 1 \( \mu \)g of His-PRMT6 (4 h of incubation), or 50 units of partially purified cellular PRMT1. 500 ng of Sm B/B' was methylated with 2 \( \mu \)g of PRMT5/His-pICL\textsubscript{10} (4 h of incubation). Proteins were separated by SDS-PAGE. PABPN1 and Sm B/B' protein bands were cut out of the gels, washed three times with water, twice with 50 mM ammonium bicarbonate, and finally with 50 mM ammonium bicarbonate in 50% acetonitrile. The gel pieces were dried under a stream of nitrogen, reswollen in 20 \( \mu \)l of 50 mM ammonium bicarbonate, pH 8.0, and digested with trypsin (Promega) overnight at 37°C. The peptide mass fingerprint spectra were recorded on an Ultraflex-III TOF/TOF mass spectrometer (Bruker) equipped with MALDI source, nitrogen laser, LIFT cell for fragment ion post-acceleration, and gridless ion reflector. The software Flex Control 2.4, Flex Analysis 2.4, and Biotools 3.0 were used to operate the instrument and analyze the data. For external calibration, a peptide calibration mixture (Bruker) was used. The methylated peptides were fragmented using the LIFT method (49). For MALDI sample preparation, 0.5 \( \mu \)l of a saturated solution of \( \alpha \)-cyano-4-hydroxyphenylacetic acid in acetone was deposited onto the sample target. 1 \( \mu \)l of the digest was added to the matrix layer. After evaporation of the solvent the spot was recrystallized with 1 \( \mu \)l of ethanol, acetone, 0.1% trifluoroacetic acid (6:3:1).

**Native Gel Electrophoresis of PABPN1–Poly(A) Complexes**—Poly(A) was size-fractionated by elution from slices of a denaturing polyacrylamide gel and \( \gamma^{32} \text{P} \)ATP (36). Labeled \( A_{\text{260}} \) (80 fmol of 3' ends) was incubated in 20 \( \mu \)l of polyadenylation buffer (25 mM Tris-HCl, pH 8.0, 50 mM KCl, 10% glycerol, 2% polyethylene glycol 6000, 0.01% Nonidet P-40, 0.05 mM EDTA, 1 mM dithiothreitol, 2 mM MgCl\(_2\), 0.4 mg/ml methylated bovine serum albumin, 0.5 mM ATP) with increasing amounts of PABPN1 (0, 160, 800, 1600, and 3200 fmol). Saturation was calculated using the assumption of 20 PABPN1-binding sites per poly(A) molecule. After 30 min of incubation at room temperature, 10 \( \mu \)l of the reaction was loaded onto a native agarose/polyacrylamide composite gel (50). The gel was dried and analyzed with a Phospho-Imager (GE Healthcare).

**Immobilization of PABPN1 and Pulldown Experiments**—NHS-activated Sepharose beads (GE Healthcare) were washed three times with 1 mM HCl. 185 \( \mu \)g of either His-PABPN1, recombinant nontagged PABPN1, PABPN1 purified from calf thymus, or bovine serum albumin was coupled separately to 185-\( \mu \)l packed volume of NHS-activated beads overnight in the cold. In 50 mM Hepes, pH 8.3, 150 mM KCl, 10% glycerol. Less than 10% of the input protein was detected in the supernatant after coupling. Residual activated groups were blocked with blocking buffer (500 mM ethanolamine, pH 8.3, 500 mM NaCl, 10% glycerol) overnight in the cold. Beads were washed three times with blocking buffer, three times with 100 mM sodium acetate, pH 4.0, 300 mM NaCl, 10% glycerol, again three times with blocking buffer, and five times with binding buffer (50 mM Hepes, pH 7.6, 150 mM KCl, 10% glycerol, 1 mM EDTA, 0.5 mM dithiothreitol). 2.3 mg of cytoplasmic or nuclear extract from HeLa cells (51) was incubated with 20 \( \mu \)l of packed volume of beads. After incubation for 4 h in the cold, the beads were washed four times with binding buffer. Bound proteins were eluted with 40 \( \mu \)l of SDS sample buffer (250 mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerine, 1.43 M 2-mercaptoethanol, 0.02% bromphenol blue). 5 \( \mu \)l of the eluted fractions were loaded onto a 10% SDS-polyacrylamide gel, and Western blot analysis was performed with a rabbit polyclonal antibody directed against PABPN1.

**RESULTS**

**PRMT1 Methylates PABPN1**—HeLa cell extracts were initially employed in an unbiased approach to identify, by protein fractionation, protein-arginine methyltransferases acting on PABPN1. We have previously described the fractionation of HeLa cell PRMTs by DEAE chromatography (8). \textit{In vitro} methylation assays of these DEAE column fractions using recombinant bovine PABPN1 as a substrate showed two peaks of methylation activity, which corresponded roughly to the two peaks of PRMT1 protein, detected by Western blotting. The two PRMT1 peaks overlapped with the peaks of PRMT3 and -6, whereas PRTM2 and -4 were present in the flow-through and in the earliest fractions, which were devoid of methylation activity (supplemental Fig. 1). When the first methylation activity peak, containing both PRMT1 and -3 but not PRMT6, was further purified by cation exchange chromatography followed by phenyl-Sepharose chromatography, active fractions were obtained that contained PRMT1 as the only detectable type I arginine methyltransferase (Fig. 1). The type II enzyme PRMT5 was undetectable as well (data not shown). Methylation activity also coeluted with PRMT1 in subsequent gel filtration (data not...
Arginine Methylation of the Nuclear Poly(A)-binding Protein

In vitro methylation

Western blot

PABPN1

PRMT1

PRMT3

FIGURE 1. PRMT1 from cytoplasmic HeLa cell extract methylates PABPN1. The enzyme was partially purified by sequential chromatography on DEAE-Sepharose, MacroPrep S, and phenyl-Sepharose as described under "Experimental Procedures." The figure shows the profile of the phenyl-Sepharose column. 10 μl of the load, flow-through (F.T.), wash (W.), and indicated column fractions were incubated for 2 h in an in vitro methylation assay (see "Experimental Procedures"). Methylated PABPN1 was visualized by phosphorimaging (top panel). For Western blots, 15 μl of the indicated fractions were loaded on an 12% SDS-polyacrylamide gel, and the elution profiles of PRMT1 (middle panel) and PRMT3 (bottom panel) were analyzed by Western blotting (see "Experimental Procedures").

FIGURE 2. Prmt1−/− ES cells have a reduced methylation activity toward PABPN1. A, 44 μg each of cytoplasmic HeLa cell extract, total wild-type ES cell extract, and total Prmt1−/− ES cell extract were incubated with His-PABPN1 (PABPN1), the deletion variant His-PABPN1 ΔC49 (ΔC49), or no protein (−) for 2 h in an in vitro methylation assay (see "Experimental Procedures"). After SDS-PAGE, the incorporation of radioactively methyl groups was visualized by phosphorimaging. The methylation activity was quantified and is indicated at the bottom, with the activity of the wild-type ES cell extract taken as 100%. B, wild-type and Prmt1−/− ES cells were incubated for 3 h in the presence of [3H]methyl-H4methylhistidine, cycloheximide, and chloramphenicol (see "Experimental Procedures"). After cell lysis, PABPN1 was immunoprecipitated with the single chain antibody 3F5 (see "Experimental Procedures"). Western blots showed that PABPN1 behaved as a single entity during purification (data not shown); thus, selective loss of a significant fraction of the protein differing in its methylation status can be excluded. Analysis of the purified material by mass spectrometry showed a major peak with a mass of 32573 Da (supplemental Fig. 2). The difference to the calculated mass (32,206 Da, assuming removal of the start methionine and acetylation of the N terminus, as found for the bovine protein (32)) was 366 Da, indicating the presence of 26 methyl groups. A second smaller peak with a mass of 32544 Da corresponded to protein containing 24 methyl groups. Because PABPN1 purified from calf thymus contains 26 methyl groups (32), the data indicate that the majority of PABPN1 in Prmt1−/− cells is fully methylated. Thus, other PRMTs can substitute for PRMT1 very efficiently.

Methylation of PABPN1 in Cells Lacking PRMT1—To confirm a role of PRMT1 in the methylation of PABPN1, we made use of a mouse ES cell line with a homozygous disruption of the Prmt1 gene (Prmt1−/− cells) (52). Extracts from these cells had an 8-fold reduced methylation activity for PABPN1, but activity was still clearly detectable (Fig. 2A). These data confirm a major role of PRMT1 but show the involvement of additional PRMTs. Methylation of PABPN1 by other PRMTs was confirmed by an experiment in which proteins were labeled in vivo by [3H]methylmethionine under conditions of translation inhibition. PABPN1 was then precipitated by the single chain antibody 3F5 (41) directed against an epitope that is not subject to methylation. Incorporation of radioactivity was readily detectable in the Prmt1 knock-out cells (Fig. 2B). As noted previously in a similar experiment (8), the background of immunoprecipitation was increased with extracts from the Prmt1 knock-out cells.

If PRMT1 is the main enzyme responsible for the methylation of PABPN1, one would expect that the latter should not be fully methylated in the Prmt1−/− ES cell line. However, we were unable to detect significant amounts of unmethylated PABPN1 with the help of an antibody specifically directed against an unmethylated PABPN1 peptide (data not shown). Also, when recombinant PRMT1 was added to extract of Prmt1−/− ES cells, endogenous PABPN1 was not methylated to any significant extent, suggesting that the protein was already methylated (data not shown). For a definitive analysis, PABPN1 was purified from these cells by column chromatography (see "Experimental Procedures"). Western blots showed that PABPN1 behaved as a single entity during purification (data not shown); thus, selective loss of a significant fraction of the protein differing in its methylation status can be excluded. Analysis of the purified material by mass spectrometry showed a major peak with a mass of 32573 Da (supplemental Fig. 2). The difference to the calculated mass (32,206 Da, assuming removal of the start methionine and acetylation of the N terminus, as found for the bovine protein (32)) was 366 Da, indicating the presence of 26 methyl groups. A second smaller peak with a mass of 32544 Da corresponded to protein containing 24 methyl groups. Because PABPN1 purified from calf thymus contains 26 methyl groups (32), the data indicate that the majority of PABPN1 in Prmt1−/− cells is fully methylated. Thus, other PRMTs can substitute for PRMT1 very efficiently.

PRMT3 and -6 Also Methylate PABPN1 but PRMT2 and -4 Do Not—Extracts of the Prmt1 knock-out cells were used to facilitate the search for additional PRMTs acting on PABPN1. Three separable activities were found, as summarized in Fig. 3. Upon DEAE chromatography, weak methylation activity was found in the wash and the first column fractions (fraction Ia), whereas a major methylation peak was eluted in later fractions of the salt gradient (fraction Iib). Fraction Ila contained...
PRMT2, -3, and -4. Because the activity profile was distinct from the PRMT2 elution profile, and no catalytic activity has been reported for this enzyme (see Introduction), PRMT2 was not further considered. Subsequent Mono S chromatography (fraction IIIa) resulted in a strong increase in methylation activity, suggesting removal of an inhibitor. The activity was distributed over one major and one minor peak, each corresponding to a PRMT3 peak. PRMT4 was separated from PRMT3 and the methylation activity (Fig. 4). The same column fractions did not methylate PABPN1ΔC49 (data not shown). These results identify PRMT3 as a second methyltransferase acting on PABPN1.

Fraction IIb of the DEAE column contained PRMT6; other type I PRMTs were not detectable in these fractions but PRMT5 was (data not shown). Further analysis of a representative DEAE column fraction by gel filtration separated two methylating activities, which were further purified by Mono Q chromatography. One of these activities (fraction IVbb; Fig. 3) closely corresponded to the elution profile of PRMT6 (Fig. 5). Residual PRMT5 was also present, but its elution profile was clearly distinct from that of the methylation activity. PABPN1ΔC49 was not methylated by the column fractions (data not shown). Thus, PRMT6 is a third enzyme modifying PABPN1. A separate activity (fraction IVba; Fig. 3) contained no detectable type I PRMTs but, surprisingly, corresponded closely to the chromatographic behavior of PRMT5 (data not shown). This enzyme is not expected to act on PABPN1, because no symmetric dimethylation was detectable in the protein (32). Accordingly, the recombinant PRMT5-pClIn heterodimer did not accept PAPBN1 as a substrate but was active in control reactions with its authentic substrates, SmD3 and SmB/B' (23) (data not shown). Attempts to determine whether PRMT5 in the column fractions was responsible for methyla-

![FIGURE 3. Fractionation scheme for methylation activities from Prmt1ES cells. Extract was fractionated by consecutive chromatographic steps as indicated (see “Experimental Procedures”). After each fractionation step, distribution of the methylation activity was tested by in vitro methylation assays, and the elution profiles of individual PRMTs were examined by Western blot analysis (see “Experimental Procedures”).](image)

![FIGURE 4. The methylation activity of fraction IIIa coelutes with PRMT3 but not with PRMT4. Fraction IIIa (see Fig. 3) was fractionated on a Mono S column as described under “Experimental Procedures.” 15 μl of the load, flow through (F.T.), wash fraction (W), and indicated column fractions were incubated for 3 h in an in vitro methylation assay (see “Experimental Procedures”). Incorporation of radioactive methyl groups was visualized by phosphorimaging (top panel). 15 μl of the indicated fractions were loaded on a 10% SDS-polyacrylamide gel, and the elution profiles of PRMT3 (middle panel) and PRMT4 (bottom panel) were analyzed by Western blotting.](image)

![FIGURE 5. The methylation activity of fraction IIIbb coelutes with PRMT6. Fraction IIIbb (see Fig. 3) was fractionated on a Mono Q column as described under “Experimental Procedures.” 10 μl of the load, flow-through (F.T.), wash fraction (W), and indicated column fractions were incubated for 3 h in an in vitro methylation assay (see “Experimental Procedures”). Incorporation of radioactive methyl groups was visualized by phosphorimaging (top panel). 15 μl of the indicated fractions were loaded on a 10% SDS-polyacrylamide gel, and the elution profile of PRMT6 was analyzed by Western blotting (bottom panel).](image)
Arginine Methylation of the Nuclear Poly(A)-binding Protein

TABLE 1
Specific activities of PRMTs under standard reaction conditions and their contributions to PABPN1 methylation

| Enzyme | Specific activity (no tag) | Specific activity (His tag) | Specific activity (cellular enzyme) | PRMT/total protein in HeLa cells | Contribution to PABPN1 methylation in HeLa cells |
|--------|---------------------------|-----------------------------|------------------------------------|---------------------------------|-----------------------------------------------|
|        | units/µg                  | units/µg                    | units/µg                           | units/µg                        | units/µg                                      |
| PRMT1  | 1460                      | 1350                        | 1450                               | 450                             | 650,000                                       |
| PRMT3  | 360                       | 200                         | 170                                | 230                             | 39,000                                        |
| PRMT6  | 120                       | 220                         | 550                                | 100                             | 55,000                                        |

The type of arginine dimethylation generated in vitro was determined after incubation of PABPN1 with recombinant PRMT1, -3, and -6. The isolated PABPN1 was digested with trypsin, and a peptide mass fingerprint spectrum was recorded. All peptide ions containing dimethylated arginine were further fragmented in the mass spectrometer. A characteristic neutral loss of m/z 45 (dimethylamine) in the MS/MS spectra was a proof of asymmetric dimethylation in all cases. (supplemental Fig. 4; supplemental Table 1). As a control, after reaction of PRMT5 with SmD and SmB/B′, symmetric dimethylarginine was detectable by a typical neutral loss of m/z 70 (dimethylcarboxydrimide) (55) (data not shown).

The partially purified cellular PRMTs, only PRMT1 was sufficiently active for this type of analysis; again, only asymmetric dimethylarginine was found (supplemental Table 1 and data not shown). These data are consistent with the exclusive presence of asymmetric dimethylarginine in PABPN1 purified from tissue (32).

PRMT1 Is the Predominant Methyltransferase for PABPN1—The abundance of PRMT1, -3, and -6 was estimated by Western blot analysis of HeLa cell extract in comparison with known quantities of recombinant polypeptides (Table 1). In agreement with earlier data (56), PRMT1 was found to be the most abundant methyltransferase. The relative contributions of the three enzymes to the methylation of PABPN1 were estimated from their abundance and their specific activities under standard conditions (Table 1). The results show that close to 90% of the total methylation activity directed against PABPN1 is because of PRMT1 because this enzyme is the most abundant and has the highest activity for this substrate. This calculated contribution of PRMT1 is in good agreement with the assays of wild-type and Prmt1−/− ES cell extract. PRMT3 and -6 contribute 5% and 7% because of a combination of lower activities and lower abundance.

Steady-state parameters for the methylation of PABPN1 by PRMT1, -3, and -6 were determined. PRMT3 activity is limited by a relatively high \( K_m \) value, whereas PRMT6 has a low \( V_{max} \) value. As a result, the catalytic efficiency of PRMT1, expressed as \( k_{cat}/K_m \), is more than 10-fold higher compared with PRMT3 and -6 (Table 2). In similar experiments, \( K_m \) values for the other substrate, S-adenosylmethionine, were 1.5, 14.2, and 1.9 µM for PRMT1, -3, and -6, respectively (data not shown).

Substrate Specificity of PRMTs—We have previously reported that the protein hnRNP K is methylated exclusively by PRMT1 (8). In agreement with these data, steady-state kinetic parameters show that methylation of hnRNPK by PRMT3 and -6 is 120- and 8-fold less efficient than methylation of PABPN1,
Arginine Methylation of the Nuclear Poly(A)-binding Protein

TABLE 2
Steady-state kinetic parameters characterizing the methylation of PABPN1, hnRNP K, and fusion proteins by PRMT1, -3, and -6

Experiments were carried out with His-tagged recombinant enzymes (see “Experimental Procedures”). The fusion proteins PABPN1-KS and -KL are described under “Experimental Procedures” and supplemental Fig. 6. Standard deviations of $V_{\text{max}}$ were generally below 10%, and those of $K_m$ were below 20%.

| Enzyme | Substrate | $V_{\text{max}}$ | $K_m$ | $k_{cat}$ | $K_m/k_{cat}$ |
|--------|-----------|-----------------|-------|-----------|--------------|
|        |           | nmol/min/mg     | $\mu$M | s$^{-1}$ | s$^{-1} \cdot$ M$^{-1}$ |
| PRMT1  | PABPN1    | 25.0            | 0.07   | 0.018     | 270,000     |
|        | hnRNP K   | 65.6            | 0.14   | 0.046     | 325,000     |
|        | PABPN1-KS | 61.2            | 0.12   | 0.043     | 356,000     |
|        | PABPN1-KL | 64.9            | 0.05   | 0.045     | 716,000     |
| PRMT3  | PABPN1    | 61.2            | 0.14   | 0.046     | 325,000     |
|        | PABPN1-KS | 5.3             | 0.25   | 0.0003    | 100         |
|        | PABPN1-KS | 16.8            | 4.57   | 0.017     | 3700        |
|        | PABPN1-KL | 10.8            | 8.71   | 0.01      | 1200        |
| PRMT6  | PABPN1    | 1.9             | 0.11   | 0.0013    | 13,000      |
|        | hnRNP K   | 2.7             | 1.23   | 0.002     | 1600        |
|        | PABPN1-KS | 4.3             | 0.09   | 0.003     | 34,900      |
|        | PABPN1-KL | 3.8             | 0.11   | 0.003     | 25,700      |

TABLE 3
Steady-state kinetic parameters characterizing the methylation of synthetic peptides by PRMT1, -3, and -6

Experiments were carried out with His-tagged recombinant enzymes (see “Experimental Procedures”). The fusion proteins PABPN1-KS and -KL are described under “Experimental Procedures”. Standard deviations of $V_{\text{max}}$ were generally below 10%, and those of $K_m$ were below 20%. Sequences of peptides were as follows: RXR-1, FYSGFNSPGRGGYATSYW, corresponding to amino acids 280–303 of bovine PABPN1 with an internal deletion of amino acids 294–298; RXR-2, FYSGFNSPGRGGRGGYATSYW, corresponding to the same sequence without the internal deletion; RXR-3, SGFSGFSGFSGFSGGRGGYATSYW, corresponding to amino acids 275–303 of bovine PABPN1. All arginines in these three peptides (underlined) are methylated in the authentic protein. RGG, GGGGFGRGGRGGFGRGGGFG, representing an RGG motif, was methylated with a high efficiency. More specifically, both fusion proteins as well as both K peptides were methylated with an efficiency comparable with that of the best protein substrate. Thus, the enzymes recognize their substrates via local amino acid sequences and not by contacting additional domains.

For a further investigation of substrate recognition by PRMT3, two fusion proteins were generated in which the methyl-accepting C-terminal domain of PABPN1 was replaced with amino acid sequences representing the domain of hnRNP K containing the methyl-accepting arginine residues (positions 256, 258, 268, 296, and 299) (8). Both domains are likely to be unstructured. The fusion protein PABPN1-KS contained full-length hnRNP K amino acids 251–309 at the C terminus, and fusion protein PABPN1-KL contained hnRNP K amino acids 241–333 in the same position (supplemental Fig. 6). As expected, both fusion proteins were excellent substrates for PRMT1 (Table 2). PRMT3 methylated both fusion proteins with reasonable efficiency. More specifically, both fusion proteins as well as both K peptides were methylated with a high $k_{cat}$, whereas poor methylation of full-length hnRNP K was limited by a very low $k_{cat}$. Unexpectedly, both fusion proteins were also very good substrates for PRMT6, being methylated 20-fold more efficiently than hnRNP K and 2–3-fold more efficiently than PABPN1 (Table 2). These data suggest that the inability of hnRNP K to serve as a substrate for PRMT3 and -6 must be due to the structure of the protein somehow impairing access of the methyltransferases.

Arginine Methylation Does Not Affect the Propensity of PABPN1 to Aggregate—Two types of experiments were carried out to determine whether arginine methylation has any influence on the tendency of PABPN1 to self-aggregate. First, three

which is relatively inefficient to begin with, whereas methyla-
tion of the two proteins by PRMT1 proceeds with similar effi-
ciencies. As a consequence, PRMT3 and -6 are 3000- and 200-
fold less efficient than PRMT1 in the methylation of hnRNP K (Table 2).

In a first attempt to investigate which features determine the substrate properties of the two proteins, synthetic peptides were used. Peptide sequences are shown in the legend to Table 3 and aligned with protein sequences in supplemental Fig. 6. The sequence of the RGG peptide corresponds to the RGG consensus and has been used as a generic methylation substrate before (57). The RXR-1 peptide sequence was modified from the C-terminal domain of PABPN1 to keep its length and arginine content directly comparable with the RGG peptide. Because this peptide turned out to be less efficient substrate than PABPN1, two longer peptides from this protein, RXR-2 and -3, were also used. Because methylated arginines in hnRNP K are spread over a larger region (8), this protein was represented by two peptides, K1 and K2. Results of methylation assays are summarized in Table 3.

For PRMT1, catalytic efficiencies obtained with the best peptides (RXR-2 and K2) were similar to those seen with the corresponding protein substrates. A slightly lower efficiency with the RXR peptides is similar to what we have observed with partial C-terminal deletions of PABPN1 with reduced numbers of arginine residues.3 In contrast, the “standard” RGG peptide was not used efficiently by PRMT1. For PRMT6, the synthetic peptides also seemed to be good representatives of the proteins from which they were derived. The RXR-2 peptide was nearly as good a substrate as PABPN1, and the two K peptides were poor substrates. The RGG peptide was also a poor substrate. For PRMT3, the RXR peptides were reasonably good substitutes for PABPN1. However, the two peptides representing hnRNP K, in particular K1, were much better substrates than the entire protein, suggesting some inhibitory effect of the environment in which these sequences are found in hnRNP K. The best substrate for PRMT3 was the RGG peptide. For every PRMT, there was at least one peptide that was methylated with an efficiency comparable with that of the best protein substrate. Thus, the enzymes recognize their substrates via local amino acid sequences and not by contacting additional domains.

3 K. Köbel and E. Wahle, unpublished data.
variants of PABPN1 were covalently immobilized on Sepharose beads as follows: PABPN1 purified from calf thymus, which is fully methylated (32); His-tagged PABPN1 purified from E. coli, and nontagged PABPN1 also purified from E. coli; the latter two proteins are both unmethylated. Bovine serum albumin was immobilized as a control. The beads were then used for a pull-down experiment. They were incubated with HeLa cell extract and washed; proteins remaining bound to the beads were eluted with SDS-containing sample buffer, separated on an SDS-polyacrylamide gel, and analyzed by Western blotting with PABPN1-specific antibodies. All three species of immobilized PABPN1 reproducibly pulled down PABPN1 from the extract, whereas the albumin control did not. Results were the same with or without micrococcal nuclease treatment of the extract; thus, the self-association of PABPN1 was not RNA-mediated (data not shown). Whereas the His-tagged immobilized PABPN1 always pulled down about 3-fold more PABPN1, there was no difference between the nontagged recombinant PABPN1 and the protein purified from calf thymus (Fig. 6A). We conclude that the His tag favors PABPN1 self-association but the absence of methylation does not.

In a second type of experiment, the same three PABPN1 variants were allowed to associate with poly(A) of a length of ~280 nucleotides, and the complexes were analyzed by native gel electrophoresis. With both calf thymus PABPN1 and nontagged recombinant PABPN1, clean ladders of bands were obtained, reflecting increasing occupancy of the polynucleotide with protein (Fig. 6B). These data confirm that arginine methylation has no effect on the RNA binding affinity of PABPN1 (33). In contrast, the His-tagged protein showed no clear banding pattern but a strongly retarded, poorly defined smear instead, suggesting nonspecific aggregation on the polynucleotide (Fig. 6B). Thus, this experiment also suggests that the His tag promotes self-association of PABPN1, whereas the lack of methylation does not. This effect of the His tag in gel shift analyses was not noticed in earlier experiments presumably because of the use of shorter RNA ligands and different buffer conditions.

With a view to a potential role of arginine methylation of PABPN1 in the fibril formation occurring in OPMD patients, we also examined whether the polyaniline expansion affects the ability of the protein to serve as a PRMT substrate. Titrations of the mutant protein in activity assays with PRMT1, -3, and -6 showed that its ability to be methylated was at most 2-fold reduced in comparison with wild-type (supplemental Fig. 5 and data not shown). This small difference is unlikely to be biologically important, given that PABPN1 methylation is almost complete even in the Prmt1−/− cell line.

**DISCUSSION**

In this work we have identified PRMT1, -3, and -6 as the enzymes responsible for arginine methylation of PABPN1. A previous study (32) was incomplete, as only PRMT1 and -3, the two enzymes then known, were tested. At the time, we suggested that PRMT3 might be the more important enzyme for the modification of PABPN1. This erroneous conclusion was because of the use of GST fusions of the PRMTs. The N-terminal GST part strongly inhibits the enzymatic activity of PRMT1 but not of PRMT3 (8). We now find that an N-terminal His tag does not affect the activities of PRMT1, -3, and -6. The enzymes partially purified from cell extracts had very similar specific activities to the recombinant enzymes, and no stimulatory factors were lost during the purification. Thus, methylation of PABPN1 is carried out by the catalytic polypeptides on their own, without the help of additional subunits. This is in contrast to the methylation of histones by PRMT1, which depends on targeting by transcriptional coactivators, and also to the methylation of Sm proteins by PRMT5, which relies on substrate recognition by the associated subunit pICln (see Introduction).

The ability of PABPN1 to be methylated by PRMT1, -3, and -6 is different from other PRMT1 substrates, like Sam68 or hnRNP K, which are methylated exclusively by this enzyme (7, 8). Thus, the substrate specificities of PRMT1, -3, and -6 are...
distinct but overlapping. This is also observed in the ability of the enzymes to use synthetic peptides as substrates. Some peptides are relatively good substrates for all three PRMTs (e.g., RXR-2), whereas others are methylated almost exclusively by one enzyme. For example, K2 is methylated by PRMT1 240-fold more efficiently in comparison to PRMT3 and 1700-fold more efficiently in comparison to PRMT6. In contrast, the RGG peptide is by far the best substrate for PRMT3 but a very poor substrate for PRMT1 and -6 (Table 3).

The preferences of the individual PRMTs for certain peptides are in good agreement with their preferences for the proteins from which the peptides are derived, and the kinetic constants characterizing the methylation of entire proteins are similar to those determined with peptide substrates. Thus, PRMT1, -3, and -6 recognize local amino acid sequences as their substrates. This is in contrast to PRMT5, in which recognition of the Sm fold outside the methylated domain is important for methylation (22–24). However, the substrate specificities of the PRMTs are not solely determined by their preferences for certain peptide sequences. Even though hnRNP K is a very poor substrate for PRMT3 and -6, sequences taken from this protein can be efficiently methylated by the two enzymes when the substrates are offered either as free peptides or as C-terminal domains appended to a carrier protein (in this case, the RGG box sequence was previously considered a good substrate for PRMT1, the major methyltransferase; several peptides deviating very strongly from the RGG box sequence were up to 40-fold better. Osborne et al. (62) also found that the RGG peptide was a relatively poor substrate compared with the histone H4 peptide. The identification of the RGG box as a consensus PRMT substrate was based mostly on the comparison of sequences surrounding methylated arginines identified in proteins. This may be misleading as the derived consensus may reflect the function of the methylated protein domain, RNA binding in the case of the RGG box, rather than the substrate specificity of the methyltransferase.

Osborne et al. (62) concluded that a patch of basic amino acid residues at the C terminus of their peptide substrate, separated by 12 amino acids from the methyl-accepting position, was important for recognition by PRMT1. This cannot be seen with the peptides tested here. In the best substrate, K2, we have not determined which of the four arginine residues is methylated, but the two most distant arginine residues are separated by only 8 amino acids, and there is no “patch” of several basic residues. In the RXR-1 peptide, still a reasonably good substrate, the middle arginine is the dominant methylation site, and with the exception of the two arginines in the immediate vicinity, there is no basic residue on either side.

The ability of PRMT1, -3, and -6 to substitute for each other in the modification of a subset of their substrates may contribute to the viability of Prmt1−/− mice (11). Even though PRMT1 appears to contribute about 90% of the total capacity of a cell to methylate PABPN1, the removal of the enzyme leads only to a very small loss in the overall methylation of this particular substrate. Thus, an investigation of the phenotypic effects of a lack of PABPN1 methylation will require RNA interference-mediated knockdown of PRMT3 and -6 in the Prmt1−/− background. The small effect of a Prmt1 deletion on the methylation level of PABPN1 is surprising in view of the low activity of the PRMTs with kcat values of 2/min or less. This low activity was not improved by the use of synthetic peptide substrates or by any of a number of additives that we tested, including poly(A), the ligand for PABPN1, or an ATP-regenerating system in a crude extract. Thus, the PRMTs seem to be intrinsically slow. We also considered the possibility of synergistic effects of multiple PRMTs in the modification of PABPN1. Because PRMT6 prefers monomethylated arginine as a substrate (15, 48), the enzyme might efficiently use products generated by PRMT1 or -3. However, in all pairwise combinations of the three PRMTs, activities were roughly additive (data not shown).

As explained in the Introduction, several pieces of evidence suggested that arginine dimethylation might reduce the inclination of proteins to associate in a presumably non-specific manner. Surprisingly, our data suggest that self-association of PABPN1 is not strongly influenced by its modification. The pulldown assays with immobilized PABPN1 clearly demonstrate the ability of the protein to form oligomers. Moreover, an increase in self-association dependent on the N-terminal His tag was easily detectable. In contrast, no influence of methylation was observed. In the formation of RNA-protein complexes, assayed by native gel electrophoresis, aggregate formation dependent on the His tag was
Arginine Methylation of the Nuclear Poly(A)-binding Protein

again evident, whereas no such effect was seen for the lack of arginine methylation. In analytical ultracentrifugation, His-tagged, nonmethylated PABPN1 sediments as a monomer at a concentration of 2 μM (29). Unfortunately, our attempts to investigate the concentration dependence of aggregation more systematically were not successful because the protein tended to aggregate in an erratic manner during the purification when present at high concentration, as reported previously (37). Thus, the effect of His tag and arginine methylation could not be examined more thoroughly.

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Arginine Methylation of the Nuclear Poly(A)-binding Protein

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