Unusual Sites of Arginine Methylation in Poly(A)-binding Protein II and in Vitro Methylation by Protein Arginine Methyltransferases PRMT1 and PRMT3*

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Arginine methylation is a post-translational modification found mostly in RNA-binding proteins. Poly(A)-binding protein II from calf thymus was shown by mass spectrometry and sequencing to contain N⁰⁰,N⁰¹-dimethylarginine at 13 positions in its amino acid sequence. Two additional arginine residues were partially methylated. Almost all of the modified residues were found in Arg-Xaa-Arg clusters in the C terminus of the protein. These motifs are distinct from Arg-Gly-Gly motifs that have been previously described as sites and specificity determinants for asymmetric arginine dimethylation. Poly(A)-binding protein II and deletion mutants expressed in Escherichia coli were in vitro substrates for two mammalian protein arginine methyltransferases, PRMT1 and PRMT3, with S-adenosyl-l-methionine as the methyl group donor. Both PRMT1 and PRMT3 specifically methylated arginines in the C-terminal domain corresponding to the naturally modified sites.

Poly(A)-binding protein II (PABP2) is a protein of 33 kDa that binds poly(A) with high affinity and specificity. PABP2 is thought to be involved in pre-mRNA polyadenylation. In vitro, it stimulates poly(A) polymerase, conferring processivity on the reaction, and is responsible for poly(A) tail-length control (1–3). The protein is composed of an acidic N termius, a ribonucleoprotein (RNP)²-type RNA binding domain in the center, and an arginine-rich C terminus (4). Both the RNP domain and the C terminus contribute to RNA binding.³

A well known arginine-rich RNA binding domain is the RGG domain. The RGG motif is defined as a variable number of closely spaced Arg-Gly-Gly (RGG) repeats interspersed with other, often aromatic amino acids (5, 6). RGG motifs are normally found in conjunction with RNP domains (7, 8) and have been shown to increase the affinity of a protein for RNA (9–13). It is possible that RGG motifs also confer specific RNA binding to a protein, since heterogeneous nuclear RNP, protein U has no other discernible RNA binding motif and can discriminate between different RNA sequences (5). Other functions of the RGG domain thus far described include mediation of protein-protein interactions (14, 15) and of nuclear localization (16, 17).

A characteristic feature of the RGG motif is the post-translational modification of arginine residues to N⁰⁰,N⁰¹-dimethylarginine (N⁰⁰,N⁰¹-DMA) (18–23). This modification is carried out by protein arginine methyltransferases (PRMTs) using S-adenosyl-l-methionine as a methyl donor. Rat PRMT1 (24) and the human homologue HRMT1L2 (25) methylate RGG motif-containing proteins and homologous synthetic peptides in vitro to give N⁰⁰,N⁰¹-DMA and/or monomethylarginine (MMA) residues. A second mammalian methyltransferase termed PRMT3 has been cloned. Although this enzyme methylates a glutathione-S-transferase (GST) fusion protein containing a glycine-and arginine-rich region from human fibrillarin, GST-GAR, no natural protein substrates have yet been identified for PRMT3 in heated hypomethylated rat cell extracts. PRMT3 is predominantly cytoplasmic, whereas PRMT1 is largely confined to the nucleus (26). Sequence comparison of mapped DMA residues showed the preferred amino acid sequence Phe/Gly-Gly-Gly-Arg-Gly-Gly/Phe with the C-terminal-flanking glycine being invariant (22). It is unclear which enzyme is responsible for the modification of this sequence in mammalian cells in vivo (see “Discussion”). In contrast, the yeast arginine methyltransferase Rmt1p is known to be responsible for arginine dimethylation in vivo (27, 28). One identified in vivo substrate is the RGG domain containing RNA binding protein Npl3p (28, 29).

The biological function of arginine methylation is unknown. A role in signal transduction has been suggested (24, 30, 31). However, the modification may be irreversible: the amino-alkyl bond is very stable, and there is no evidence of a DMA demethylase. Other suggested functions include modulation of intracellular trafficking of macromolecules (32–34) and of protein binding to RNA (35). A yeast rmt1 deletion strain is viable under standard growth conditions (27, 28).

Upon amino acid analysis of PABP2 from calf thymus, only

³ A. Nemeth, U. Kühn, and E. Wahle, unpublished data.
50% of the expected arginine residues were found (4). Here we show that arginine is methylated at 15 locations in PABP2. Almost all modified arginines are located in the C terminus of the protein in sequence motifs distinct from previously described sites of arginine methylation. The mammalian arginine methyltransferases PRMT1 and PRMT3 preferentially methylate the C terminus of recombinant PABP2 in vitro.

**Experimental Procedures**

**Purification of Proteins**—PABP2 was purified from calf thymus as described (3). PABP2 used for enzymatic digestion was further purified on a Nucleosil 500–5 C18-HPLC column (150 × 5 mm; Nucleosil, Du¨ ren, Germany) equilibrated with 0.09% trifluoroacetic acid and eluted with a 0–100% gradient of the same buffer containing 1 M KCl. PABP2-con- (Amersham Pharmacia Biotech) in dialysis buffer and eluted with a 0.3–60% solvent B (0.08% trifluoroacetic acid in aceto- nitrite) gradient over 25 min with a flow rate of 0.2 ml/min at 40 °C. The identity of the PABP2 peak was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the fraction was dried under nitrogen. Cysteine residues were alkylated with vinylpyridine as described (36). The alkylated protein was desalted by HPLC and dried as above. GST-PRMT fusion proteins were purified as described (24, 26).

**Preparation of Recombinant PABP2 and Deletion Mutants**—The PABP2 coding sequence from a pT7–7-PABP2 construct (4) was cloned into BamHI sites of the pGM10/Hisα expression vector (37). From this construct a fusion protein with the sequence Met-Ala-Hisα-PABP2 was expressed. Deletion mutants were obtained through polymerase chain reaction using pT7–7-PABP2 as a template and suitable oligonucleotide primers. PABP2 C2 contained PABP2 amino acids 1 through 257, PABP2 ΔN α amino acids 161 through 306, RNP domain amino acids 161 through 257, and the C-terminus amino acids 258 through 306. Polymerase chain reaction products were cut with NdeI and BamHI and cloned into pGEM10/Hisα so that each protein was expressed with the N-terminal sequence Met-Ala-Hisα. Constructs were checked by sequencing. Proteins were expressed in Escherichia coli BL21-pLys-S in Superbroth medium. 2-liter cultures with an A600 of around 2 were induced with 0.4 mM isopropyl β-D-thiogalactopyranoside and grown for 4 h at 37 °C. Cells were pelleted, stored overnight at –80 °C, and suspended in 50 ml of lysis buffer (50 mM Tris-HCl, pH 8.0, 100 mM KCl, 10% (v/v) glycerol). This and all subsequent buffers contained 0.5 mM phenylmethylsulfonyl fluoride, 0.4 mM di-thiothreitol. Proteins were loaded onto a Mono S column (Amersham Pharmacia Biotech) in dialysis buffer and then with 10 ml of lysis buffer containing 10 mM imidazole. PABP2-containing fractions were dia lyzed against 25 mM Tris-HCl, pH 8.0, 100 mM KCl, 10% (v/v) glycerol. This and all subsequent buffers contained 0.5 mM phenethylsulfonfluoride, 0.4 µg/ml leupeptin, and 0.7 µg/ml pepstatin. Lysates were prepared by sonication for 5 min, cleared by centrifugation, and incubated for 2 h with 1 ml nickel nitrotriacetic acid–agarose (Qiagen) pre-equilibrated with lysis buffer. The slurry was loaded into a column and washed with 10 ml of lysis buffer and then with 10 ml of lysis buffer containing 10 mM imidazole. Hisα-tagged proteins were eluted with 5 × 1-ml fractions of lysis buffer containing 250 mM imidazole. PABP2-containing fractions were dia lyzed against 1 liter of 25 mM HEPES, pH 7.9, 10% glycerol, 10 mM KCl, 0.5 mM dithiothreitol. Proteins were loaded onto a Mono S column (Amersham Pharmacia Biotech) in dialysis buffer and eluted with a 0–100% gradient of the same buffer containing 1 mM KCl. PABP2-con taining fractions were dia lyzed against 25 mM Tris-HCl, pH 8.0, 10% glycerol, 10 mM KCl, 0.5 mM dithiothreitol, and frozen in liquid nitrogen.

**Enzymatic Digestion of PABP2**—Sequencing grade proteases were obtained from Roche Molecular Biochemicals. Lys-C digestion of PABP2 was carried out in 50 µl of 25 mM Tris-HCl, pH 8.5, with a Lys-C to PABP2 ratio of 1:200 (w/w) overnight at 37 °C. Digests were separated by HPLC as above with a 0–40% solvent B gradient over 60 min and a flow rate of 0.2 ml/min at 40 °C. Fragments labeled Lys-C1–8 in the order of elution (Table I) were dried under a nitrogen stream and their masses, and assignments, see Table I. Sequences obtained from individual fragments are listed in Table II.

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**RESULTS**

**Location of Modified Arginine Residues in PABP2 from Calf Thymus**—We set out to identify the nature of arginine modifications and to map their locations in the entire PABP2 sequence by proteolytic digestion of the protein and subsequent analysis of the derived fragments by sequencing and MALDI-TOF-MS. Lys-C fragments that could be assigned to the PABP2 sequence were labeled Lys-C1–8 in the order of elution from the HPLC column (Table I). The fragments designated Lys-C5b and Lys-C5c were shouldered eluting after the main peak Lys-C5a. The entire PABP2 sequence could be accounted for except for three small peptides (Leu-136—Lys-137, Phe-208—Lys-213 and Val-244—Lys-247), none of which contained arginine. The cDNA-derived PABP2 amino acid sequence with the sites of modification is shown in Fig. 1. All residue numbering is according to this sequence. For an overview of the fragments, their masses, and assignments, see Table I. Sequences obtained from individual fragments are listed in Table II.

**Fifteen sites of arginine modification were found in the remaining PABP2 fragments by mass spectral analysis and sequencing. The largest discrepancy between observed and predicted mass was seen in the C-terminal fragment Lys-C6, corresponding to Arg-248—Tyr-306. The difference, 364.8 Da of peptide-derived pyridylethyl cysteine modifications, indicates that the two arginine residues present are unmethylated.**

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hydantoin derivatives of the modified arginines had the same retention time in the sequencer as an \( N^{\text{Ac}} \) standard. Sequences obtained from two secondary chymotryptic fragments, one generated by incomplete digestion, confirmed the dimethylation of arginines 287, 289, 291, 294, 296, and 298 (Table II).

Some heterogeneity in the extent and nature of arginine modification was observed at two locations. The two peptides Lys-C5b and Lys-C5c, eluted from the HPLC column as shoulderers of Lys-C5a, were, based on their masses, assigned to Glu-224—Lys-243 with the addition of 1 and 2 methyl groups, respectively. The predicted sequence of the peptides (ESVRTS-LALDESFRGRQK) contained three arginine residues, any one of which could be a site of modification. Sequencing confirmed the identity of the peptides and identified their fourth amino acid as unmodified arginine. Data quality was not sufficient for unequivocal identification of the remaining arginines. To localize the site of modification, a secondary tryptic digest was carried out on peptides Lys-C5b and Lys-C5c. Mass spectral analysis of the entire digest gave fragments with masses corresponding to the predicted sequence TSLALDES- FRGR with the addition of one and two methyl groups for Tryp-Lys-C5b and Tryp-Lys-C5c, respectively. Trypsin will not cleave after a methylated arginine residue (22), and therefore it is assumed that the site of modification in both Lys-C5b and Lys-C5c is Arg-238. In the Lys-C5b tryptic digest, a significant peak with a mass of 1251 Da, corresponding to Thr-228—Arg-238 without modification, was also observed, indicating that the separation of the two peptides Lys-C5a and Lys-C5b was not complete. The relative amounts of the unmodified, monomethylated, and dimethylated peptides, i.e. Lys-C5a, Lys-C5b, and Lys-C5c, were approximately 2:1:1 based on HPLC peak heights.

Fragment Lys-C8, corresponding to the N terminus of the protein, had an observed mass of 11,836.7 Da. The predicted mass was 11,930.7 Da. The N terminus of the protein was blocked, and the mass discrepancy infers removal of the initial methionine residue and N terminus acetylation. However, the high mass of this peak made discernment of partial modification with a single methyl residue unlikely, so a secondary tryptic digest was carried out. Fragments obtained from this digest (Tryp-Lys-C8) included minute amounts of two peptides corresponding to the sequence acetyl—Ala-2—Arg-23 with the addition of 1 or 2 methyl groups. The full predicted sequence of these fragments was acetyl-AAAAAADAGAAAGGRGS- PGR. As in the case of Lys-C5b and Lys-C5c, the presence of methylated arginine groups in a tryptic peptide ending with PGR. As in the case of Lys-C5b and Lys-C5c, the presence of methylated arginine groups in a tryptic peptide ending with PGR.
arginine infers that the modification occurs on the interior arginine, in this case Arg-17. Further evidence was obtained by treatment of the entire tryptic digest with carboxypeptidase B. Mass spectral analysis after this treatment gave fragments with masses of the original fragments less the mass of one unmodified arginine residue. In contrast to Arg-238, which was modified to about 50%, a very small proportion (<5%) of Arg-17 was found to be methylated. We cannot be certain as to the exact identity of the modified residues at Arg-17 and Arg-238, as these fragments could not be sequenced. However, as N5-N7-DMA (symmetric) has thus far only been found in myelin basic protein (23, 38), it is highly unlikely that the modified arginine residues at Arg-17 and Arg-238 are the symmetric form of DMA. Arg-24 and Arg-25 could not be assigned to any tryptic fragment from the Tryp-Lys-C8 digest. This suggests that they are unmodified arginine residues that served as tryptic cleavage sites. It is also possible that Arg-24 is modified to a very small extent, and Arg-23 and Arg-25 serve as tryptic cleavage sites. The resulting dipeptide Arg-24—Arg-25 would not be detected by MALDI-TOF-MS.

The measured mass of the entire HPLC-purified protein was 33,253 Da. This is in excellent agreement with the predicted mass of 33,252.5 Da, assuming removal of the initial methionine, acetylation, alkylation of two cysteine residues, and the addition of 26 methyl groups.

E. coli-expressed PABP2 and Deletion Mutants Are Substrates for the Arginine Methyltransferases PRMT1 and PRMT3—The two mammalian methyltransferases PRMT1 and PRMT3, expressed in E. coli as GST fusion proteins (24, 26), were able to methylate E. coli-expressed PABP2 and various deletion mutants with S-adenosyl-L-methionine as the methyl group donor (Fig. 2). As expected, the best substrates were those that contained the C terminus of the protein. Both the N-terminal deletion mutant (PABP2 ΔN) and the isolated C-terminal domain (Fig. 2, lanes 4 and 5) appeared to be better substrates for both PRMT1 and PRMT3 than the complete protein (lane 1). In the experiment shown in Fig. 2, use of PRMT3 at an approximately 2.5-fold higher molar concentration than PRMT1 led to a similar extent of methylation. In previous experiments (26), PRMT3 had approximately 1% of the activity of PRMT1 when GST-GAR was used as a methyl-accepting substrate. Thus, the activity of the GST-PRMT3 protein toward PABP2 was higher than expected. Extra methylated bands in Fig. 2, b and c, are presumably contaminants from the substrate or PRMT fusion protein purification. Their identities are unknown.

Lys-C digestion of full-length recombinant PABP2 methylated in vitro by GST-PRMT1 followed by MALDI-TOF-MS analysis indicated a shift of the C-terminal fragment to a higher mass (Fig. 3, b and d). No additional peaks corresponding to methylated species were found in any other peptide (compare with Fig. 3, a and c); all observed masses were in close agreement with predicted values (data not shown). The C-terminal peak was centered at around 7069 mass units, corresponding to the addition of four methyl groups per fragment. The mass distribution of the C-terminal fragment could be modeled with an average of 0.22 methyl groups/arginine residue (data not shown). Taking into account this level of methylation, the resolution of MALDI-TOF MS and the number of arginines in the different peptides, and assuming random methylation, methylated species of the peptides corresponding to Lys-C2, Lys-C7, and Lys-C8 should have been detectable. These species were not observed, indicating that methylation in vitro was preferentially directed to the C terminus. Preferential methylation of the C terminus upon in vitro methylation of PABP2 by GST-PRMT3 was also observed (data not shown).

**DISCUSSION**

Previous studies on the substrate specificity of arginine methylation, based on sequence comparison of the 20 thus far mapped sites of arginine dimethylation, described a preferred recognition motif of Phe/Gly-Gly-Gly-Arg-Gly-Gly/Phe with the C-terminal-flanking glycine considered obligatory (22, 23, 39). In PABP2, only 3 of the 13 identified sites of complete arginine dimethylation have a C-terminal-flanking glycine, and of these three, none have an N-terminal-flanking glycine. Arg-17 has N- and C-terminal-flanking glycine residues, but only a small subpopulation of Arg-17 is mono- or dimethylated. Twelve of the modified residues occur within a distinct RXR motif, where both arginines in the motif are completely asymmetrically dimethylated, and X is, in most cases, a small amino acid (Gly, Ala, Ser, or Pro) or, in one case, tyrosine.

PABP2 contains three RXR motifs (Arg-23—Arg-25, Arg-76—Arg-78, and Arg-125—Arg-127) with unmethylated argin-
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The sequence RXXR is thus not a sufficient determinant of arginine dimethylation. As amino acids 76–78 are RPR, the identity of X cannot be a sufficient determinant either. It remains to be determined whether the additional criteria are the sequence context, the accessibility of the arginines for the methyltransferase, or the clustering of RXXR motifs. The N-terminal deletion mutant was a better substrate for both PRMT1 and PRMT3 than the entire protein. It is possible that the acidic N terminus makes intra- or intermolecular contacts with the basic C terminus, the disruption of which improves accessibility for the methyltransferase. Note, however, that the increased molar concentration of the smaller substrates may also have promoted increased levels of methylation.

A database search for other proteins containing an RXXRXRXR sequence with Pro, Tyr, Ala, or Gly in the X position identified a number of nuclear proteins including high molecular weight basic fibroblast growth factor and ribosomal protein S2. High molecular weight basic fibroblast growth factor has been shown to contain DMA within its RG motifs (32, 40, 41), although the exact nature of the arginine modification has yet to be established. Ribosomal protein S2 has RGGF and RGR motifs and is likely to contain DMA (42). ICP27, an RNA-binding protein from herpesvirus, has RGR repeats in its sequence and is methylated in vivo (43). Although Arg-238 is only partially methylated, Arg-240 is unmethylated, this observation suggests that RGR motifs in the C terminus of PABP2 might also form type I β-turns.

The finding that two different sequences, RXXR clusters and (F/G)GRG(G/F) (see above), are subject to asymmetric arginine dimethylation might suggest that each of the two identified mammalian arginine methyltransferases serves one of these two substrate types. However, the situation is far from clear. Most studies of arginine methylation in vitro have been carried out with enzyme preparations from mammalian tissue (23, 39, 46, 47). Their molecular compositions were ill-defined, and their relationship to PRMT1 and -3 is unknown. The two cloned methyltransferases, PRMT1 and PRMT3, may be just the catalytic subunits of larger complexes (23). Moreover, in vitro arginine methyltransferase reactions are generally plagued by poor efficiency. Thus, conclusions concerning substrate specificity may be premature. The activity of both PRMT1 and PRMT3 in vitro was directed toward the C terminus of PABP2, with PRMT3 apparently being more efficient than expected from previous experiments with a standard RGG substrate. This suggests that both enzymes may have specificity for RXXR clusters. There is a possibility that this apparent specificity is directed by the structure of the substrate protein rather than a preference of the enzymes for particular amino acid sequences. However, in a comparison of several synthetic peptides, a peptide with clustered RGR sequences was by far the best in vitro substrate for arginine methyltransferase preparations from three different tissues (38).

Biochemical assays of PABP2 have so far not revealed any function for arginine methylation. Recombinant and authentic proteins are indistinguishable with respect to the stimulation of polyadenylation and length control (4). Under standard reaction conditions, we have also not detected any difference in the RNA binding properties of the two proteins.3

In addition to some similarity in the general amino acid composition, the C-terminal RXXR domain of PABP2 shares two further features with the RGG domain in that it contains the...
characteristic modified amino acid asymmetric dimethylarginine, and it binds RNA in a nonsequence specific manner. Structural studies are needed to decide whether R and RGG sequences are merely two variations on the same theme or two different motifs fulfilling similar roles.

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