Arginine Methylation of the Nuclear Poly(A) Binding Protein Weakens the Interaction with Its Nuclear Import Receptor, Transportin

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Background: Arginine dimethylation in proteins has been reported to affect nuclear import.
Results: Arginine methylation of the nuclear poly(A) binding protein in the binding site for its import receptor, transportin, weakens the interaction about 10-fold.
Conclusion: We hypothesize that a delay of methylation until after import may contribute to the directionality of import.
Significance: This may be a general mechanism of regulating nuclear import.

The nuclear poly(A) binding protein, PABPN1, promotes mRNA polyadenylation in the cell nucleus by increasing the processivity of poly(A) polymerase and contributing to poly(A) tail length control. In its C-terminal domain, the protein carries 13 arginine residues that are all asymmetrically dimethylated. The function of this modification in PABPN1 has been unknown. Part of the methylated domain serves as nuclear localization signal, binding the import receptor transportin. Here we report that arginine methylation weakens the affinity of PABPN1 for transportin. Recombinant, unmethylated PABPN1 binds more strongly to transportin than its methylated counterpart from mammalian tissue, and in vitro methylation reduces the affinity. Transportin and RNA compete for binding to PABPN1. Methylation favors RNA binding. Transportin also inhibits in vitro methylation of the protein. Finally, a peptide corresponding to the nuclear localization signal of PABPN1 competes with transportin-dependent nuclear import of the protein in a permeabilized cell assay and does so less efficiently when it is methylated. We hypothesize that transportin binding might delay methylation of PABPN1 until after nuclear import. In the nucleus, arginine methylation may favor the transition of PABPN1 to the competing ligand RNA and serve to reduce the risk of the protein being reexported to the cytoplasm by transportin.

Asymmetric dimethylation of arginine side chains, a common posttranslational modification of proteins in eukaryotic cells, is carried out by type I protein arginine methyltransferases (PRMTs2 1, 2, 3, 4, 6 and 8), which use S-adenosyl-methionine as the methyl group donor. Although the rules of PRMT substrate recognition are unclear, dimethylated arginine residues often occur clustered in sequences that are likely to lack stable secondary or tertiary structures. Like other protein modifications, arginine methylation serves to regulate protein-protein interactions (1–7).

Many arginine-methylated proteins reside in the cell nucleus, and several studies support a role of the modification in nucleocytoplasmic transport. For example, when protein methylation in mammalian cells is prevented by nonspecific chemicals, inhibition of methyltransferases or genetic inactivation of individual PRMTs, several nuclear proteins relocalize to the cytoplasm (8–15). Cytoplasmic localization of other proteins appears to be favored by arginine methylation rather than its inhibition (16–19). A direct effect of arginine methylation on protein localization is supported by the fact that the amino acid sequences containing the methyl-accepting arginines either correspond to nuclear localization signals (NLSs) or otherwise affect cellular localization in most of the cases cited above. A direct effect was proven by experiments in which nuclear import of RNA helicase A was prevented by methylation inhibitors and could be rescued by in vitro methylation of the protein prior to injection (12). In yeast, the RNA binding proteins Npl3p, Nab2p, and Hrp1p are substrates for the only type I PRMT of yeast, Hmt1p, and their nuclear export depends on arginine methylation. Genetic evidence suggests that Npl3p itself needs to be methylated. In contrast, Hmt1p appears to affect the nuclear export of Hrp1p indirectly via methylation of Npl3p (19–22). The methylation-sensitive molecular interactions affecting nucleocytoplasmic transport remain largely unknown. In the case of yeast Npl3p, arginine methylation interferes with phosphorylation of a particular serine residue that is required for efficient interaction with the import receptor of the protein, Mtr10p (23). Coprecipitation experiments showed that arginine methylation of the receptor interacting protein 140 favors its interaction with the nuclear export receptor exportin 1 (24).
The mammalian nuclear poly(A) binding protein (PABPN1) is implicated in 3' end processing of pre-mRNA in the nucleus. Binding the growing poly(A) tail, PABPN1 stimulates the activity of the poly(A) polymerase and also limits processive polyadenylation to a length of approximately 250 nucleotides (25–28). In support of this model of PABPN1 function, a mutation in the gene encoding the *Drosophila* orthologue leads to a reduction of poly(A) tail length (29). PABPN1 consists of an acidic N-terminal domain, an RNA recognition motif-type RNA binding domain, and an arginine-rich C-terminal domain (30, 31). All 13 arginine residues within the C-terminal domain are quantitatively asymmetrically dimethylated (32). PRMT1, PRMT3, and PRMT6 are able to methylate PABPN1 *in vitro* (33). Although the C-terminal domain contributes to both poly(A) binding and stimulation of polyadenylation, neither function is affected by the modification (30). The PABPN1 orthologue in *Schizosaccharomyces pombe*, Pab2, has an increased aggregation tendency in the absence of arginine methylation (34), but this does not appear to be the case for mammalian PABPN1 (33). Interestingly, the C-terminal domain of PABPN1 is necessary and sufficient for the nuclear localization of the protein. On the basis of its RanGTP-sensitive binding, the likely receptor for nuclear import is transportin (karyopherin B2) (35).

Transportin is a member of the importin-β family and directs the nuclear import of many RNA-binding proteins. Directed transport by importin-β-type import receptors depends on the asymmetric distribution of RanGTP. A low RanGTP concentration in the cytoplasm permits binding of import substrates, whereas a high RanGTP concentration in the nucleus favors its association with the import receptor and displacement of imported cargo (36–38). Biochemical and structural analyses have identified a consensus sequence for NLSs recognized by transportin. The signals are structurally disordered with an overall basic character and a length of 20–30 amino acids. The consensus sequence is composed of three parts: a C-terminal highly conserved PY dipeptide, separated by two to five amino acids from a more N-terminally located basic residue and, again toward the N terminus, a basic or hydrophobic patch (39–43).

Looking for a biological function of arginine methylation in PABPN1, we have examined the interaction of the protein with transportin. We report that methylation significantly weakens the PABPN1-transportin interaction and suggest that the modification may serve to promote the steady-state localization of PABPN1 in the cell nucleus.

**EXPERIMENTAL PROCEDURES**

**Proteins, Synthetic Peptides, and Antibodies—**His-tagged PABPN1, His-tagged PABPN1-ΔC8, PABPN1 lacking a tag, and His-tagged PRMT1 were expressed in *Escherichia coli* and purified as described (30, 33). Calf thymus PABPN1, purified to homogeneity, was the preparation described in Ref. 33. The PABPN1 mutant C195/205S, in which both cysteine residues were replaced by serine, was generated by site-specific mutagenesis. For the fusion of protein A with PABPN1-C195/205S, the cysteine mutant was digested with Xho1 and BamH1, and the resulting fragment was used to replace the correspond-

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The PABPN1-NLS peptides corresponded to the last 25 amino acids of human or bovine PABPN1. In one version of the peptide, all arginine residues were asymmetrically dimethylated, and the other version was unmethylated. Both peptides contained free N and C termini and were synthesized by Peptide Specialty Laboratories GmbH, Heidelberg, Germany.

The antibody against transportin was a kind gift of Ulrike Kutay, Swiss Federal Institute of Technology (ETH Zürich) (45). The single chain llama antibody 3F5 recognizes a PABPN1 epitope between amino acids 113 and 133 (47, 48).

**Cell Culture and Preparation of Nuclear Extract—**HeLa cells were cultured in Dulbecco's modified Eagle’s medium (Invitrogen) with 10% fetal bovine serum and 1% penicillin/streptomycin solution (Invitrogen). ES cells were cultured as described (49). Nuclear cell extracts were prepared as described (50), but the dialysis step was omitted.

**Pull-down Experiments—**Pull-down experiments were carried out essentially as described (33). Briefly, proteins were covalently immobilized on NHS-activated Sepharose beads (GE Healthcare) (5 μg of PABPN1 variant or 2.5 μg of transportin per 5 μg of beads). Successful coupling was checked by the absence of protein in the supernatant after coupling. Beads were then incubated either with HeLa cell nuclear extract or recombinant proteins as indicated in buffer A (50 mM HEPES (pH 7.9), 20% glycerol, 1 mM EDTA, 0.5 mM dithiothreitol) containing salt as indicated and 1.25 mg/ml bovine serum albumin. After incubation, beads were washed with buffer A containing 150 mM KCl, and bound proteins were eluted with SDS sample buffer (250 mM Tris-HCl (pH 6.8), 4% SDS, 20% glycerin, 1.43 M 2-mercaptoethanol, 0.02% bromphenol blue). The eluate was analyzed by SDS-PAGE and Coomassie staining or immunoblotting with antibodies as indicated.

For the pull-down experiments with zz-transportin1, ES cell nuclear extract was treated with 0.5 U micrococcus nuclease (Fermentas) per μg protein for 42 min at 26 °C. 3 μg of nuclear extract protein was diluted to 1.2 ml with buffer A containing 420 mM NaCl and incubated with 1 μM zz-transportin1 for 1 h in the cold. Then, 54 μl of IgG-Sepharose beads (GE Healthcare) resuspended in buffer A containing 420 mM NaCl were added and incubated for an additional 2 h in the cold. beads were washed with buffer A containing 150 mM KCl, and bound proteins were eluted with GTP-loaded RanQ69L (2.2 μM), precipitated with methanol-chloroform, and analyzed by SDS-polyacrylamide gel electrophoresis and Coomassie staining or immunoblotting with antibodies as indicated. For protein identification, protein bands were cut out of SDS-polyacrylamide gels and analyzed by LC-MS/MS as described (51).
RNA Binding Assay—Nitrocellulose filter binding assays (30) were performed in 40 μl with 10 fmol (0.25 nM) 5’ end-labeled A14, 50 nM PABPN1, and transportin (0–4000 nM) in filter binding buffer (50 mM Tris-HCl (pH 8.0), 100 mM KCl, 1 mM EDTA, 0.5 mM dithiothreit, 10% glycerol, 0.2 mg/ml methylated BSA, 0.01% Nonidet P-40). RNA and PABPN1 were incubated for 15 min at room temperature and for a further 20 min after the addition of transportin. Nitrocellulose filters were incubated in wash buffer (50 mM Tris-HCl (pH 8.0), 100 mM KCl, 1 mM EDTA) with 0.5 μg/ml E. coli rRNA. 35 μl of each sample were applied to the filter on a vacuum filtration device and washed with 5 ml of ice-cold wash buffer. Bound radioactive RNA was quantified in a liquid scintillation counter.

In Vitro Methylation—In vitro methylation of PABPN1 was performed with His-PRMT1 (33). The reaction was quantified by the use of [14C]S-adenosyl-L-methionine as the methyl group donor, followed by SDS gel electrophoresis and autoradiography.

In Vitro Nuclear Import Assays—His-ProtA-PABPN1-C195/205S and the corresponding ΔC25 deletion variant were fluorescence-labeled with fluorescein 5’ maleimide at a molar ratio of 1:2 on ice for 30 min. Unbound dye was removed by gel filtration over a Nap5 column (GE Healthcare). BSA coupled to the SV40 NLS (52) was labeled with Texas Red NHS ester (Molecular Probes, Inc.).

In vitro import reactions were performed essentially as described (45). Briefly, HeLa-cells were grown on coverslips to 60–80% confluence, washed with ice-cold 1X PBS (pH 7.4) and permeabilized by 40 μg/ml digitonin in permeabilization buffer (20 mM HEPES (pH 7.5), 110 mM potassium acetate, 5 mM magnesium acetate, 250 mM sucrose, 0.5 mM EGTA) for 10 min. Cells were washed three times in permeabilization buffer and incubated with 10 μl of import mix for the time indicated at room temperature. The import mix consisted of an energy regenerating mixture (0.5 mM ATP, 0.5 mM GTP, 10 mM creatine phosphate, 50 μg/ml creatine kinase) and Ran mix (53) in import buffer (20 mM HEPES (pH 7.5), 80 mM potassium acetate, 5 mM magnesium acetate, 250 mM sucrose, 0.5 mM EGTA). Cells were washed with permeabilization buffer, fixed with 4% paraformaldehyde, washed with 1X PBS, and mounted in ProLong Antifade (Invitrogen). Images were acquired either on an Axiovert 200 m microscope (Zeiss) or a TCS SP5 laser scanning microscope (Leica) using a ×63 objective (Plan Apo ×63, Leica) and standardized settings for sequential imaging of DAPI and FITC. For quantification, integral fluorescence of imported protein was normalized to integral fluorescence of DAPI staining. For each experiment, 70 cells from two independent images were quantified, and statistical significance was examined by Student’s t test.

RESULTS

Arginine Methylation of PABPN1 Lowers the Affinity for Transportin—PABPN1 contains 13 asymmetrically dimethylated arginine residues within its C-terminal domain (32). This region is necessary and sufficient for nuclear localization of PABPN1, and transportin was suggested to be the import receptor (35). Since then, amino acid sequences recognized by transportin have been well characterized (see introduction), and indeed sequences corresponding to the consensus motif bound by transportin are found within the last 20–30 C-terminal residues of PABPN1: a basic cluster followed by the sequence RX6PY (Fig. 1A). Because both parts of the motif contain arginine residues that are asymmetrically dimethylated in vivo, the effect of methylation on transportin binding was tested. PABPN1 purified from calf thymus, which is dimethylated at all 13 arginine residues in the C-terminal domain, two variants of recombinant unmethylated protein, and BSA as a control were immobilized on Sepharose beads and incubated with nuclear extract. After washing, bound proteins were eluted and probed for transportin by Western blotting (Fig. 1B). Both batches of unmethylated PABPN1 bound transportin from the extract, whereas binding to methylated PABPN1 was almost undetectable. Unspecific protein binding to immobilized BSA was not observed. Binding was insensitive to micrococcal nuclease treatment of the extract, thus transportin binding was independent of RNA (data not shown). This experiment suggests that arginine methylation of PABPN1 decreases the affinity for transportin.

To test whether transportin itself discriminates between unmethylated and methylated PABPN1 or whether other components of the extract are involved, we incubated immobilized methylated and unmethylated PABPN1 with recombinant transportin1. Bound transportin was eluted and analyzed by Coomassie staining. Again, transportin bound unmethylated but not methylated PABPN1 (Fig. 2). Thus, methylation directly affects the interaction between the two proteins. Two criteria were used to ascertain the specificity of the interaction. First, a functional import complex should be sensitive to disso-
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**FIGURE 2. Recombinant transportin binds specifically to PABPN1 and prefers unmethylated PABPN1.** Recombinant non-methylated His-tagged and non-tagged PABPN1 (his tag, no tag), fully methylated PABPN1 from calf thymus (c.t), the recombinant non-methylated deletion variant PABPN1-ΔC8 (ΔC8), and BSA were covalently immobilized on NHS-activated Sepharose beads. Each immobilized protein (5 μg, 0.3 μM for PABPN1) was incubated with recombinant transportin 1 (6 μg, 0.12 μM) in 500 μl of binding buffer A containing 350 mM KCl, 5 mM MgCl₂ and 1.25 mg/ml BSA for 2 h at 8 °C. The salt concentration used corresponds to that introduced by the non-dialyzed high-salt nuclear extract used in Fig. 1. Where indicated, RanQ69L loaded with GTP (0.5 mM) was added to the reactions. Beads were washed, and bound proteins were eluted with SDS and analyzed by SDS-polyacrylamide gel electrophoresis and Coomassie staining.

**FIGURE 3. Transportin binding of PABPN1 is sensitive to in vitro methylation.** Recombinant PABPN1 was methylated in vitro (+ SAM) or mock-methylated (- SAM). For each time point, the reaction contained 1 μg His-PRMT1, 1 μg His-PABPN1 and, when present, 6 nmol of S-adenosyl-L-methionine in a volume of 25 μl. An additional reaction was carried out with 5 nmol of S-adenosyl-L-methionine and 1 nmol [1⁴C]-S-adenosyl-L-methionine under otherwise identical conditions. Reactions were stopped after incubation for 0, 15, 30, 60, or 120 min by the addition of 15 nmol of S-adenosyl-L-homocysteine and freezing in liquid nitrogen. The reaction containing the [1⁴C]-labeled methyl group donor was used for quantification of methylation. The extent of methylation is indicated at the top. 100% methylation corresponds to 26 methyl groups incorporated per PABPN1 molecule. For binding experiments, immobilized transportin 1 (2 μg, 0.1 μM) was incubated with PABPN1 (0.3 μg, 0.05 μM) from the various time points of the “cold” methylation reaction (left) or from the mock reaction (right). Binding reactions were carried out as in Fig. 2 in a volume of 200 μl. Beads were washed, and bound proteins were eluted with SDS and analyzed by SDS-polyacrylamide gel electrophoresis and immunoblotting with the single chain antibody 3F5 (upper panel). Equal input of all binding experiments was analyzed by immunoblotting with the same antibody (lower panel).

**In Vitro Methylation of PABPN1 Decreases Transportin Binding**—In the experiments reported so far, differences in transportin binding between methylated and unmethylated PABPN1 were tested with PABPN1 derived from different sources, recombinant versus calf thymus protein. To confirm that the effects observed were caused by methylation and not by other differences between the protein preparations, we methylated recombinant PABPN1 with PRMT1 in vitro and examined its binding to immobilized transportin. The incorporation of methyl groups was quantified by autoradiography in a parallel assay with radioactive S-adenosyl-L-methionine (SAM). PABPN1 bound by transportin was analyzed by Western blotting with an antibody that recognizes an epitope of PABPN1 outside its methylated domain (single chain antibody 3F5). An increase in methylation correlated with a decrease in transportin binding (Fig. 3). Mock methylation of PABPN1 in the absence of SAM did not change its binding affinity. Thus, the reduction in transportin binding is directly mediated by methylation of PABPN1. It is important to note that modification of the 13 methyl-accepting arginine residues is not random in vitro. Arg-289, located in the basic cluster forming part of the transporting binding site, is the preferred methylation site for PRMT1. ³ Presumably, this is the explanation why partial methylation of PABPN1 was sufficient to reduce transportin binding to almost background.

**The Unmethylated NLS of PABPN1 Efficiently Competes with PABPN1 for Transportin Binding**—The experiment shown in Fig. 1 was carried out with non-dialyzed high-salt nuclear extract. Therefore, the subsequent transportin binding experiments (Figs. 2 and 3) were also carried out at a corresponding, unphysiologically high salt concentration (350 mM KCl). Similar pull-down assays under more physiological conditions (150 mM KCl) revealed no difference in transportin binding between methylated and unmethylated PABPN1 (data not shown). Presumably, at physiological salt concentrations, the overall affinity of the PABPN1-transportin interaction is so high that differences between the two forms of PABPN1 are not detectable in the pull-down assays. Competition assays were used to confirm that arginine methylation also affects the interaction at physiological salt concentrations: Two peptides comprising the C-terminal 25 amino acids of PABPN1 were synthesized, one in the presence of increasing amounts of the peptides. The beads were washed, and transportin bound by the full-length protein was eluted and analyzed by Coomassie staining. Both peptides competed with

³ K. Köbel and E. Wahle, unpublished data.
the PABPN1-transportin interaction. Although a 2-fold molar excess of the unmethylated peptide over immobilized PABPN1 reduced binding of transportin to approximately 50%, a 10-fold higher concentration of the methylated peptide was necessary to achieve a similar competition (Fig. 4). Thus, at physiological salt concentration, arginine methylation decreases the affinity for transportin approximately 10-fold.

Arginine Methylation Affects the Competition between Transportin and RNA for Binding to PABPN1—The contribution of the C-terminal domain of PABPN1 to RNA binding (30) suggests the possibility that RNA and transportin might compete for interaction with PABPN1. To test this, nitrocellulose filter binding assays with PABPN1 and radioactive oligo(A) were performed in the presence of transportin. Indeed, PABPN1 bound less RNA in the presence of increasing transportin concentrations (Fig. 5A). Methylation has a barely detectable effect on the affinity of PABPN1 for RNA (30). The corresponding binding experiments were repeated and yielded apparent $K_d$ values of $7 \pm 4 \text{ nM}$ and $3 \pm 1 \text{ nM}$ for binding of unmethylated and methylated PABPN1, respectively, to A$_{15}$ (data not shown). Therefore, the difference between methylated and unmethylated PABPN1 in the affinity for transportin should be reflected in a corresponding difference in their sensitivities to transportin competition in RNA binding experiments. As predicted, the competition was 20-fold less pronounced when methylated PABPN1 was used (Fig. 5A). These results demonstrate that RNA and transportin binding are indeed competitive and confirm that methylation reduces the affinity of PABPN1 for transportin.

Transportin Inhibits Methylation of PABPN1—To test whether transportin binding competes with arginine methylation, PRMT1-dependent in vitro methylation of PABPN1 was performed in the presence of increasing transportin concentrations and visualized by autoradiography. Quantification showed that transportin decreased methylation of PABPN1 in a concentration-dependent manner (Fig. 5B). The competition was specific because it was reduced by the addition of GTP-loaded RanGTP. This suggests that binding of unmethylated PABPN1 to transportin prevents methylation until dissociation of the protein is achieved. In contrast, we have reported previ-
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The effect of arginine methylation on nuclear import was analyzed if transportin in fact acts as the nuclear import receptor for PABPN1. By previous studies (35) and ours show that PABPN1 binds transportin in a RanGTP-sensitive manner. To analyze if transportin in fact acts as the nuclear import receptor for PABPN1, in vitro import assays with digitonin-permeabilized cells were carried out. Because small molecules up to 40 kDa can diffuse through the nuclear pore complex, PABPN1 (32 kDa) was fused to protein A. For fluorescence microscopy, the cysteine residue of the fusion protein was labeled with fluorescein. Both cysteines of PABPN1 were mutated to serine (ProtA-PABPN1 C195/205S) to restrict labeling to the protein A part. PABPN1 carrying the double point mutation has normal RNA-binding affinity and a normal stimulatory effect in polyadenylation (data not shown). In import assays, PABPN1 nuclear accumulation was dependent on transportin (Fig. 6 and 7A). Transportin-mediated import of PABPN1 lacking the NLS (ProtA-PABPN1 C195/205S−ΔC25) was not observed, and PABPN1 was not imported by importin αβ, the functionality of which was demonstrated by nuclear uptake of the SV40-NLS fused to BSA. Thus, nuclear import of PABPN1 is specifically mediated by transportin, and the C-terminal 25 amino acids are required for nuclear import.

The effect of arginine methylation on nuclear import was tested in experiments in which increasing amounts of the methylated and unmethylated NLS peptides were used as competitors for the nuclear import of PABPN1. Although both peptides inhibited the nuclear uptake of PABPN1, the unmethylated peptide was effective at lower concentrations (Fig. 7B). Despite considerable experimental variability, quantitation of the nuclear accumulation of PABPN1 and normalization to DAPI-staining confirmed the stronger competition of the unmethylated peptide (Fig. 7C). We conclude that the difference in transportin affinity caused by arginine methylation of PABPN1 affects the efficiency of nuclear import.

FIGURE 6. Nuclear import of PABPN1 is mediated by transportin. A, 0.5 μM FITC-labeled PABPN1 or PABPN1ΔC25 were preincubated for 10–12 min under import conditions as described under “Experimental Procedures” with or without 0.75 μM transportin. Import reaction mixtures were then incubated with permeabilized HeLa cells for 18 min at room temperature. Cells were fixed with 4% paraformaldehyde, and import was analyzed by fluorescence microscopy. B, 0.5 μM FITC-labeled PABPN1 or Texas Red-labeled BSA-NLS were preincubated for 10–12 min under import conditions as described under “Experimental Procedures” with or without 0.75 μM Importin α and 0.5 μM Importin β. Import reaction mixtures were then incubated with permeabilized HeLa cells for 18 min (FITC-PABPN1) or 6 min (BSA-NLS) at room temperature. Cells were fixed with 4% paraformaldehyde, and import was analyzed by fluorescence microscopy.

FIGURE 7. Nuclear import of PABPN1 is efficiently competed by the unmethylated PABPN1-NLS. A, 0.5 μM FITC-labeled PABPN1 was preincubated for 10–12 min under import conditions with or without 0.3 μM transportin as described under “Experimental Procedures.” Import reaction mixtures were then incubated with permeabilized HeLa cells for 18 min at room temperature. Cells were fixed with 4% paraformaldehyde, stained with DAPI, and import was analyzed by confocal microscopy. B, the reactions as in A were carried out in the presence of 0.2 or 1 μM methylated (left panel) or unmethylated (right panel) NLS peptide. C, the import of PABPN1 for each experiment was quantified for 70 cells as described under “Experimental Procedures.”
nuclear extracts from wild-type mouse ES cells and from ES cells homozygous for a disruption of the predominant methyltransferase, Prmt1 (61). Following binding to IgG-Sepharose beads, transportin-interacting proteins were specifically eluted with GTP-loaded RanGTP, precipitated with methanol-chloroform, and analyzed by SDS-PAGE and immunoblotting with indicated antibodies. Unspecific binding to Sepharose beads was analyzed in the absence of zz-transportin (−). For each immunoblot, the eluate and input was quantified by the ImageQuante software (GE Healthcare). The fraction of eluate compared with input (in %) is indicated at the bottom of each immunoblot. The band just below FUS is probably a degradation product of zz-transportin, which reacts with the antibody because of its tag.

FIGURE 8. PRMT1 knockout increases binding of cellular FUS/TLS to transportin. Nuclear extracts of ES wild-type (ES +/+) or ES-PRMT1 knockout (ES −/−) cells were supplemented with zz-transportin (+) and incubated with IgG-Sepharose beads as described under "Experimental Procedures.” Bound proteins were washed, eluted by GTP-loaded RanQ69L, precipitated with methanol-chloroform, and analyzed by SDS-PAGE and immunoblotting with indicated antibodies. Unspecific binding to Sepharose beads was analyzed in the absence of zz-transportin (−). For each immunoblot, the eluate and input was quantified by the ImageQuante software (GE Healthcare). The fraction of eluate compared with input (in %) is indicated at the bottom of each immunoblot. The band just below FUS is probably a degradation product of zz-transportin, which reacts with the antibody because of its tag.

DISCUSSION

Numerous indications exist that arginine methylation can affect intracellular trafficking of proteins (see introduction), but no coherent picture has emerged so far. Also, because it has been largely unclear which physical interactions pertaining to intracellular transport are sensitive to methylation, few mechanistic models have been offered to explain the effects observed. Here, we report that arginine methylation within the transportin binding site of PABPN1 weakens the interaction approximately 10-fold. Furthermore, transportin competes with RNA for binding to PABPN1, and methylation of PABPN1 has a marginally positive effect on RNA binding. Thus, in a competitive situation, methylated PABPN1 has an approximately 20-fold higher relative affinity for RNA as compared with the unmethylated protein. Transportin also competes with access of methyltransferases to PABPN1, inhibiting in vitro methylation by PRMT1. As several other transportin cargo molecules are known to be arginine-methylated within the NLS, and as our preliminary data indicate that methylation of FUS/TLS may also decrease its affinity for transportin, the phenomenon described here is likely not to be limited to PABPN1.

Modulation of binding affinity by arginine methylation within the transportin binding site is consistent with structural data. The transportin binding site can be divided into three parts. In two subclasses of transportin cargo, the N-terminal motif is a cluster of either basic or hydrophobic amino acids. PABPN1 possesses an arginine-rich basic cluster, although hydrophobic amino acids are also present (Fig. 1A). Toward the C terminus, there is a basic amino acid, usually arginine (60), and, at a distance of two to five amino acids, the conserved PY dipeptide. Cocystal structures show that amino acids separating the three motifs can follow different paths, but the positions of the conserved motifs converge on the transportin surface. Arginine side chains present within the motifs are specifically recognized by transportin, and replacement of arginine residues by other amino acids weakens the interaction (40–42, 60). Nevertheless, the approximately 10-fold reduction in affinity caused by methylation is surprisingly large, considering that mutations in individual amino acids of a transportin-dependent NLS almost invariably have quite modest effects (40, 41, 43, 60). As the transportin binding epitope contains at least six arginine residues, it is likely that the 10-fold effect is composed of smaller contributions from methylation of multiple side chains. Cooperation between arginines has been observed (60).

What predictions can be made for the in vivo situation? Considering the high affinity of newly synthesized, unmethylated PABPN1 for transportin; the likely sequestration of the competing ligand, poly(A), by the cytoplasmic poly(A) binding protein; the large number of methyl groups to be added and the sluggish and distributive activity of the PRMTs (33, 67); and the inhibitory effect of transportin binding on PABPN1 methylation; we hypothesize that newly synthesized PABPN1 in the cytoplasm rapidly associates with transportin and is imported into the cell nucleus in a (partially) unmethylated state. In the nucleus, dissociation of the transportin-PABPN1 complex is favored by Ran-GTP and the competing ligand poly(A). Once PABPN1 has been released from transportin, it
can be methylated as RNA binding does not interfere with arginine methylation (33). Methylation favors the association with poly(A) at the expense of transportin binding. In support of this hypothesis, PRMT1, the predominant type I arginine methyltransferase overall (68) and apparently also for PABPN1 (33, 69), has been described as mostly nuclear in several studies (70, 71), although, depending on the cell type and the splice variant examined, the cytoplasm is also populated to different extents (9, 72, 73). Both PRMT1 and PRMT3 associate with PABPN1 in the nucleus (69).

Directionality of nuclear import of proteins is determined by the RanGTP gradient. Passage through the nuclear pore itself is a passive, reversible process. Therefore, to the extent to which it can bind cargo in the cell nucleus and escape the dissociating effect of RanGTP, any nuclear import receptor must also be able to function in export. At least in some cases, this is probably the basis for the shuttling behavior observed for nuclear proteins (36, 37, 74). In the simplest model, the rates of nuclear transport overall (68) and apparently also for PABPN1 (33, 71), although, depending on the cell type and the splice variant examined, the cytoplasm is also populated to different extents (9, 72, 73). Both PRMT1 and PRMT3 associate with PABPN1 in the nucleus (69).

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