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Arginine Methylation of RNA Helicase A Determines Its Subcellular Localization*

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RNA helicase A (RHA) undergoes nuclear translocation via a classical import mechanism utilizing karyopherin beta. The nuclear transport domain (NTD) of RHA is known to be necessary and sufficient for its bi-directional nuclear trafficking. We report here that arginine methylation is a novel requirement for NTD-mediated nuclear import. Nuclear translocation of glutathione *S*-transferase (GST)-NTD fusion proteins is abrogated by arginine-methylation inhibitors. However, *in vitro* arginine-methylation of GST-NTD prior to injection allows the fusion protein to localize to the nucleus in the presence of methylation inhibitors. Removal of the arginine-rich C-terminal region negates the effects of the methylation inhibitors on NTD import, suggesting that methylation of the NTD C terminus the relieves the cytoplasmic retention of RHA. The NTD physically interacts with PRMT1, the major protein arginine methyltransferase. These findings provide evidence for a novel arginine methylation-dependent regulatory pathway controlling the nuclear import of RHA.

Arginine di-methylation, which is restricted to eukaryotic cells, occurs frequently in the context of RGG tripeptides (1, 2). Two classes of protein arginine methyltransferases (PRMT)[1] have been characterized and classified based on the symmetry of their reaction products. Type I PRMTs account for the formation of asymmetric N^G,N^G-dimethylarginine, whereas type II enzymes catalyze the formation of symmetric N^G,$N^{G'}$-dimethylarginine. Five related enzymes that catalyze asymmetric arginine methylation have thus far been identified. The majority of Type I PRMT activity in eukaryotic cells appears to be accounted for by PRMT1 and its functional yeast homologue Hmt1/Rmt1 (3). Several recent studies have illustrated an important function of arginine methylation in the regulation of protein function. Methylation of Sam68, a proline-rich src-kinase substrate known to interact with WW or Src homology 3 domain containing signaling proteins, decreases its affinity for Src homology 3 domains but does not alter binding to WW motif-containing proteins (4). Similarly, arginine methylation of the STAT1 (signal transducer and activator of transcription) transcription factor decreases its affinity for its inhibitor PIAS1 (protein inhibitor of activated STAT 1), thereby modulating interferon-induced gene transcription (5). A positive modulatory effect of arginine methylation on protein-protein interaction is observed with the Type II PRMT substrates SmD1 and SmD3, which require methylation for efficient binding to the spinal muscular atrophy gene product, SMN (6, 7).

Proteins within eukaryotic cells that shuttle across the nuclear envelope to facilitate proper cell function undergo nucleocytoplasmic transport by utilizing specific nuclear transport receptors, which ferry the cargo proteins through the nuclear pore complex (NPC). A signal sequence domain contained within the cargo proteins allows for the recognition of the protein by the transport receptor (8). The classical and by far most well characterized nuclear localization sequence (NLS), which consists of multiple basic amino acids including lysine and arginine residues, was originally identified in the SV40 large T antigen (9). This NLS, which utilizes an importin α/β heterodimer as the import receptor, is both necessary and sufficient for protein transport across the NPC. During the import process, importin α binds the NLS-containing protein and serves as a link to importin β, which in turn interacts with the NPC. Recently, several proteins containing arginine-rich motifs within their NLS have been discovered that can bind to importin β independently of importin α (10, 11).

RNA helicase A (RHA), whose nuclear import was previously thought to employ importin α and β, contains several arginine residues within its nuclear localization signal sequence. Mammalian RHA, also known as nuclear DNA helicase II, was first identified as a protein capable of unwinding double-stranded RNA as well as double stranded DNA sequences and has since been shown to fulfill multiple roles in mammalian cells (12–14). As such, RHA has been implicated in transcription by binding to promoter-proximal sequences (15), bridging to the CBP/p300 complex (16) and interacting with RNA polymerase II (17). An additional function of RHA, which has similarity to yeast pre-mRNA splicing factors prp2, prp16, and prp22 (12), appears to be in cellular RNA splicing and processing through binding to the heterogeneous nuclear ribonucleoproteins K, C1, and A as well as small nuclear ribonucleoprotein (14).

RHA has also been reported to increase retroviral RNA transcription by binding to the HIV trans-activation response elements (18, 19). Additionally, RHA promotes the export of mRNA transcripts through binding to TAP and HAP95 (20, 21) and plays a role in the export of constitutive transport element-containing viral transcripts (22, 23). Because of these multiple roles of RHA in the cell, it is important to elucidate the mechanism which governs the nucleo-cytoplasmic distribution of RHA.

The C terminus of RHA contains a 110-amino acid bi-directional nuclear transport domain (NTD) that is necessary and sufficient for both nuclear import and export (24). Hetero-

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[1] The abbreviations used are: PRMT, protein arginine methyltransferase; STAT, signal transducer and activator of transcription; NPC, nuclear pore complex; NLS, nuclear localization sequence; RHA, RNA helicase A; NTD, nuclear transport domain; GST, glutathione *S*-transferase; FBS, fetal bovine serum.

karyon analysis demonstrated that the RG/RGG-rich NTD confers bi-directional shuttling activity when linked to GST or other non-transport proteins, even though immunofluorescence studies revealed that the NTD confers a primarily nuclear steady-state localization onto the fusion protein.

To date, phosphorylation is the only signal transduction mechanism shown to be involved in nuclear import. Since the NTD of RHA contains several RG/RGG motifs, we decided to investigate whether these residues are methylated by PRMT1 and whether the nuclear translocation of RHA is altered depending on its methylation state. Indeed we find that the NTD of RHA can be methylated by PRMT1 *in vitro* and that this methylation is necessary for NTD nuclear translocation. As such, our findings provide the first direct evidence that arginine methylation within a mammalian NLS regulates the function of the nuclear import signal.

MATERIALS AND METHODS

Tissue Culture and PRMT Inhibitors—U266 cells were grown in 10% fetal bovine serum (FBS). For microinjection experiments, HeLa cells were grown to 75% confluence on sterile cover slips in Dulbecco's modified Eagle's medium, 10% FBS at 37 °C. To inhibit PRMT activity cells were treated with 2 mM adenosine, 2 mM DL-homocysteine, and 2 mM N^6-methyl-2′-deoxyadenosine for 4 h at 37 °C.

Cloning, Expression, and Purification of NTD Fusion Proteins—All RHA-NTD sequences were cloned into the pGEX4T-1 vector. Protein expression was induced with 0.2 mM isopropyl-β-D-thiogalactopyranoside for 4 h at 30 °C. GST fusion proteins were purified using glutathione-Sepharose beads (Amersham Biosciences), and eluted with 20% glutathione (100 mM Tris-HCl, pH 8.0, 100 mM NaCl).

In Vitro Arginine Methylation Assays—Reactions were carried out for 90 min at 30 °C in buffer containing 20 mM Tris-HCl, pH 8.0, 0.2 M NaCl, 4 mM EDTA, 15 μg of substrate, 5 μg of GST-PRMT1, and 2 μl of *S*-adenosyl-L-[*methyl*-^{3}H]methionine (specific activity of 55.0 Ci/mmol). Reactions were stopped by adding SDS loading buffer. Proteins were subject to SDS-PAGE and transferred to polyvinylidene difluoride membrane. After blocking in 5% nonfat dry milk and soaking for 30 min in Amplify reagent (Amersham Biosciences), the blots were subjected to fluorography for 18 h at −80 °C on Hyperfilm MP (Amersham Biosciences).

Preparation of Arginine-methylated NTD for Microinjection—For these experiments, His-tagged PRMT1 was utilized instead of GST-PRMT1. Methylation reactions were incubated for 24 h at 30 °C and contained 20 mM Tris-HCl, pH 8.0, 0.2 M NaCl, 4 mM EDTA, 150–200 μg of GST-NTD, 150–200 μg His-PRMT1 on nickel beads, and 72 μM *S*-adenosyl-L-methionine. The beads were pelleted, and the supernatants containing methylated GST-NTD were concentrated using a Centricon Y-10 filtration unit (Amicon) at 5000 × *g* to a protein concentration of 10 mg/ml. To determine efficiency of the methylation stochiometry, a parallel reaction was carried out as above, except radiolabeled *S*-adenosyl-L-[*methyl*-^{3}H]methionine was used in place of non-radiolabeled SAM. Reactions were stopped by adding 800 μl of 10% trichloroacetic acid and 100 μg of γ-globulins as carrier. After trichloroacetic acid precipitation the formic acid solubilized pellets were subjected to liquid scintillation counting in a Beckman LS-6500 scintillation counter.

GST Pull-down Assays—U266 cells were lysed in 20 mM HEPES, 0.1% Nonidet P-40, 50 mM NaCl, 50 mM NaF, 10 mM β-glycerophosphate, and 1 mM phenylmethylsulfonyl fluoride. GSH beads with 25 μg of "bait protein" were added to the cell lysates and incubated overnight at 4 °C. The beads were washed five times with lysis buffer prior to addition of 50 μl of SDS loading buffer. Resolved proteins were probed for the presence of PRMT1 or RHA. To determine that the "bait" proteins were present in equal amounts, the blots were reprobed with GST antiserum.

Microinjection Assays and Immunofluorescence—HeLa cells were injected with the respective GST-NTD fusion proteins and rhodamine-conjugated dextran (Molecular Probes). Injections were performed at room temperature, and cells were returned to 37 °C after injection. After 1 h, the cells were fixed with 4% paraformaldehyde, blocked with 10% FBS, 0.2% Triton X-100 and washed four times with phosphate-buffered saline. Cells were incubated with GST antisera followed by fluorescein isothiocyanate-conjugated goat anti-mouse antibodies and fixed in mounting medium containing 4′,6-diamidino-2-phenylindole stain.

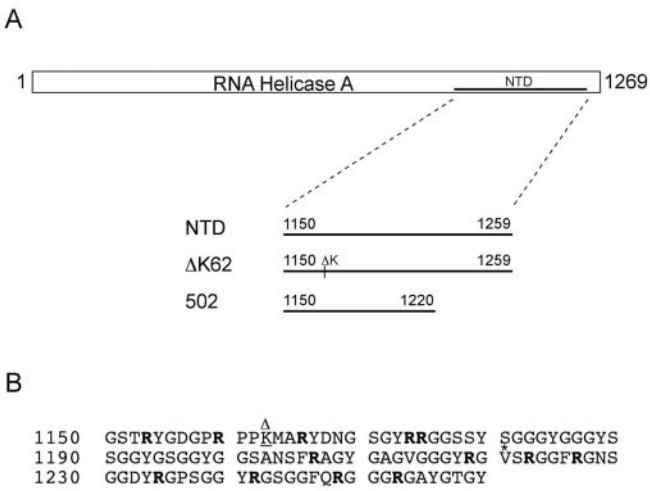

FIG. 1. **Structure of RNA helicase A.** *A*, overview of RHA structure, NTD localization, and NTD deletion mutations. *B*, 110 amino acid sequence of the NTD. RG/RGG repeats are in *bold*, K62 is *underlined*, and the asterisk marks the last residue of 508.

RESULTS AND DISCUSSION

RHA contains a 110-amino acid (position 1150–1259) bi-directional NTD that is necessary and sufficient for both nuclear import and export of the enzyme. This region harbors an NLS as well as a nuclear export sequence. Mutation or deletion of Lys-1162 causes the loss of RHA nuclear import, whereas deletion of amino acids 1221–1259 impairs the nuclear export of the protein (Fig. 1*A*). The "RGG" motifs found in the arginine-rich sequences of the NTD follow the consensus substrate recognition site for PRMTs (Fig. 1*B*). As PRMT1 accounts for the vast majority of PRMT activity in the cell, we decided to test whether the NTD can indeed function as a substrate for this enzyme. As shown in Fig. 2, PRMT1 is able to methylate GST fusion proteins of the full-length NTD (*lane 1*) as well as of both the ΔK62 and 508 deletion mutants (*lanes 2* and *3*, respectively), whereas GST alone was not methylated under these conditions (*lane 4*).

We next wanted to determine whether the NTD can interact with endogenous PRMT1 *in vivo*. Using the GST-NTD fusion proteins as bait, we performed pull-down experiments in U266 whole cell lysates. As expected, PRMT1 associated with the GST-fusion proteins of the full-length NTD as well as the ΔK62 deletion mutant but not with GST alone. Intriguingly, PRMT1 also did not bind to the GST-508 fusion protein, even though this protein is methylated by PRMT1 *in vitro* (Fig. 2*B*, *lanes 3*, *4*, and *6*). It thus appears that the C-terminal domain of the NTD, which harbors multiple RG/RGG motifs that are absent in the 508 truncation, may be crucial for the high affinity NTD/PRMT1 interaction required to sustain the association under the experimental conditions. To confirm the association of PRMT1 with RHA, we also performed pull-down experiments with GST-PRMT1 to test for its ability to associate with full-length endogenous RHA (Fig. 2*C*). Indeed, RHA bound to GST-PRMT1 but not to GST alone, supporting the concept of a physical interaction between these two proteins.

The above experiments established that PRMT1 associates with RHA and is able to methylate full-length NTD as well as the ΔK62 and 508 deletion mutants *in vitro*. The localization of the RGG motifs suggested that methylation of the arginine within the NTD might exert a regulatory effect on the subcellular localization of RHA. To test this hypothesis, we performed microinjections of the full-length GST-NTD as well as the GST-ΔK62 and GST-508 deletion mutants, followed by immunofluorescence analysis with anti-GST antibodies. Previous studies

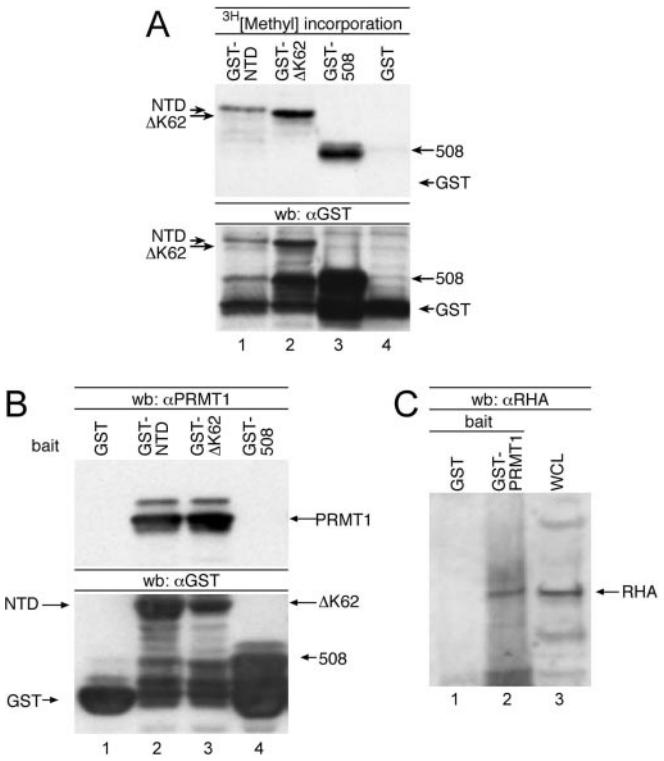

FIG. 2. **PRMT1 interacts with full-length RHA and RHA-NTD.** *A*, GST fusion proteins of full-length NTD, ΔK62, and 508 or GST alone were subject to *in vitro* methylation using recombinant PRMT1 (*upper panel*). The blot was probed with GST antisera to determine protein loading. *B*, wild-type and mutant GST-NTD fusion proteins were incubated with whole cell lysates and bound proteins probed for the presence of PRMT1 (*upper panel*). The blot was reprobed with GST antisera to determine the amounts of GST fusion proteins. *C*, whole cell lysates were incubated with GST-PRMT1 or GST alone and associated proteins probed for the presence of RHA.

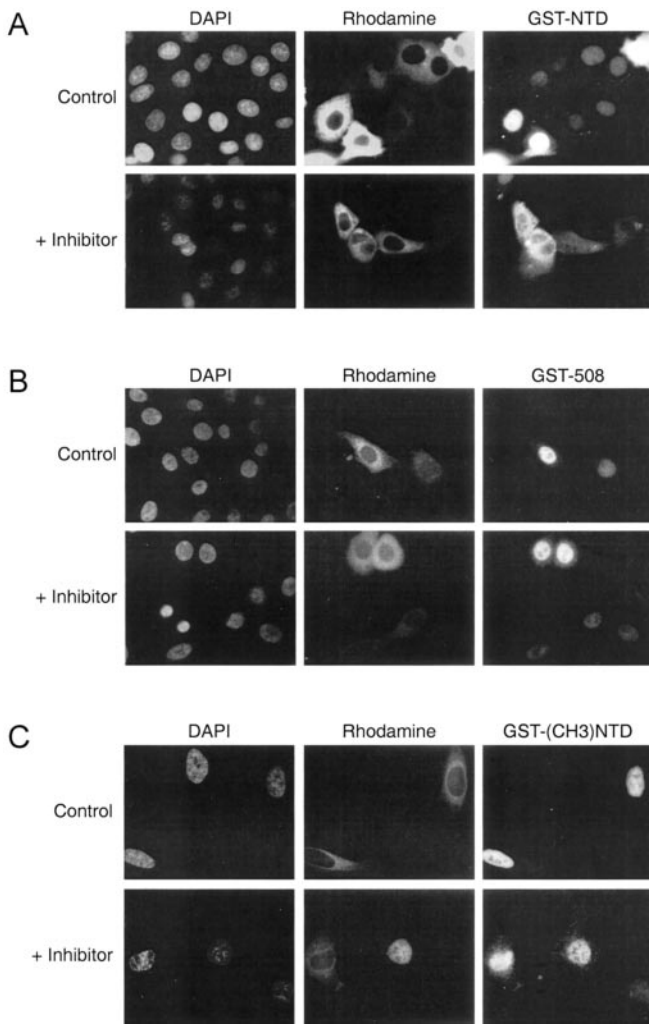

FIG. 3. **Arginine methylation is required for NTD-mediated nuclear import.** *A*, GST-NTD was injected cytoplasmically into untreated cell (*upper panels*) or into cells treated with PRMT inhibitors as described under "Materials and Methods" (*lower panels*). Rhodamine-dextran was employed as an injection site marker, and 4′,6-diamidino-2-phenylindole was used to stain nuclei. Localization of the injected GST protein was determined by immunostaining with GST antiserum. *B*, same as A, except GST-508 was microinjected. *C*, same as in *A*, except that the GST-NTD fusion protein was *in vitro* methylated by PRMT1 prior to microinjection.

had demonstrated that the GST-NTD fusion protein displays a nuclear steady-state localization despite its shuttling between cytoplasm and nucleus (24). Accordingly, when full-length GST-NTD was injected into the cytoplasm of HeLa cells, it appeared in the nucleus in 100% of the injected cells (Fig 3*A*, *top row*). To determine whether arginine methylation would alter the subcellular localization of GST-NTD, we treated the cells with PRMT inhibitors prior to microinjection of the GST-NTD fusion protein. Incubation of cells with adenosine and DL-homocysteine in the presence of the *S*-adenosylhomocysteine-hydrolase inhibitor *N*-methyl-2-deoxyadenosine results in the cellular accumulation of *S*-adenosylhomocysteine, a potent inhibitor of PRMTs. We had previously found this regimen to be highly effective in abrogating the arginine methylation of the interferon activated transcription factor STAT1 (25). When these methylation inhibitors were added to cells prior to microinjection, more than 85% of the injected cells displayed a clear inhibition of nuclear import of the GST-NTD (Fig. 3*A*, *bottom row*). Importantly, when the truncated GST-508 fusion protein, which lacks several of the RGG motifs, was injected, it localized to the nucleus even in the presence of the methylation inhibitors (Fig. 3*B*). Together, these findings not only demonstrate that the RGG-motifs in the C terminus of the NTD, which are absent in GST-508, are not necessary for the nuclear localization but also argue that arginine methylation of its C-terminal region overcomes a negative effect exerted by this region on the nuclear import of the GST-NTD.

One concern with the interpretation of the above experiments is the possibility that the abrogation of GST-NTD nuclear import by the PRMT inhibitors might not be due to blocking of the methylation of the NTD itself. Even though the fact that the nuclear translocation of the GST-508 is unaffected by the methylation inhibitor argues against this possibility, we could not exclude that the methylation inhibitors do not only target the methylation of the NTD but also that of additional cofactor(s) that might be required for its nuclear translocation. We therefore decided to subject the GST-NTD to *in vitro* arginine methylation by recombinant PRMT1 prior to microinjection of such arginine methylated fusion protein into untreated cells or into cells that had previously been exposed to the methylation inhibitors. As shown in Fig. 3*A*, unmethylated GST-NTD is restricted to the cytoplasm when injected into cells treated with the methylation inhibitors. In contrast, when *in vitro* arginine-methylated GST-NTD is introduced into these cells, we found that the premethylated GST-NTD was still able to localize to the nucleus (Fig. 3*C*). These results clearly establish that the arginine methylation of the NTD itself is a crucial requirement for the nuclear import of the NTD fusion protein. The above results and those of additional nuclear and cytoplasmic microinjections are summarized in Table I. To exclude the possibility that our observations were an artifact of the isolated

TABLE I
Summary of microinjections of wild-type and mutant NTD fusion proteins

Wild-type and mutant NTD GST fusion proteins were microinjected into the cytoplasm (Cyto) or nucleus (Nuc) of untreated cells or into cells treated with PRMT inhibitors. Site of injection, presence of PRMT inhibitors, and localization of the fusion proteins are listed.

Construct	Injection	Drug	Localization
NTD	Cyto	No	Nuc
NTD	Cyto	Yes	Cyto
NTD	Nuc	No	Nuc
NTD	Nuc	Yes	Nuc
CH3-NTD	Cyto	No	Nuc
CH3-NTD	Cyto	Yes	Nuc
ΔK62	Cyto	No	Cyto
ΔK62	Cyto	Yes	Cyto
ΔK62	Nuc	No	Nuc
ΔK62	Nuc	Yes	Nuc
508	Cyto	No	Nuc
508	Cyto	Yes	Nuc
508	Nuc	No	Nuc
508	Nuc	Yes	Nuc

NTD, we performed heterokaryon assays with GFP-RHA(ApaI) as previously described (24). Exposure of the cells to methylation inhibitors prevented the movement between the nuclei as anticipated (data not shown).

The role of phosphorylation in the control of nuclear import in mammalian cells has been well documented; in contrast, a function for arginine methylation as a requirement for this process has not been previously reported. Precedence exists for a role of arginine methylation in cytoplasmic nuclear transport in *Saccharomyces cerevisiae*. There, the RGG-rich domain of Npl 3p, when methylated by Hmt1/Rmt1, inhibits the phosphorylation of its NLS and its nuclear import. In a converse manner, arginine methylation is known to regulate the nuclear export of heterogeneous nuclear ribonucleoproteins in yeast.

Our results presented here provide the first evidence of arginine methylation-dependent nuclear import in mammalian cells. Consistent with a role for arginine methylation in NTD-mediated nuclear import, we find that the RHA-NTD can be methylated *in vitro* by PRMT1. Moreover, the NTD interacts with PRMT1 in GST pull-down assays, and conversely, GST-PRMT1 is able to bind endogenous RHA. Nuclear import mediated by the NTD of RHA is abrogated in the presence of inhibitors of PRMTs. This block of nuclear translocation can be overcome by *in vitro* methylating of the NTD prior to microinjection. Our studies summarized in Table I, however, do not provide any evidence for a role of arginine methylation in NTD-mediated nuclear export. Intriguingly, GST-508, created by removal of the RGG-rich C terminus of the NTD, is able to enter the nucleus even in the presence of inhibitors of arginine methylation.

One potential explanation for the differential dependence of RHA-NTD and RHA-508 on arginine methylation for their nuclear localization might be the presence of an unidentified cytoplasmic "retention factor," which binds to the unmethylated RGG-rich domain of the NTD and thereby prevents access of importin β to the NTD-NLS. Arginine methylation of the NTD causes the inhibitory protein to be released from RHA and thus allows for the exposure of the NLS, a process that is abrogated by inhibition of the methylation reaction. Removal of the RGG-rich C-terminal domain of the NTD eliminates the docking site for this putative inhibitor protein and allows nuclear import to proceed even in the presence of the PRMT inhibitors. This model is consistent with the finding that PRMT1 binds to GST-NTD but not to GST-508.

In summary, we have shown that arginine methylation affects NTD-mediated nuclear import via an RGG-rich NLS; thus, it is likely that methylation affects the import of other proteins containing arginine-rich nuclear localization signals as well. As such, methylation may prove to be an important posttranslational modification of proteins requiring nuclear import/export for proper cellular function.

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