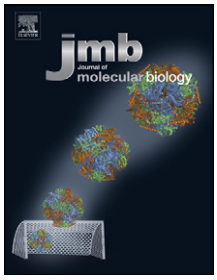

Methylarginines within the RGG-Motif Region of hnRNP A1 Affect Its IRES *Trans*-Acting Factor Activity and Are Required for hnRNP A1 Stress Granule Localization and Formation

Michael L. Wall [1] **and Stephen M. Lewis** [1,2,3,4]

*1 - **Atlantic Cancer Research Institute,** Moncton, New Brunswick, Canada*
*2 - **Department of Microbiology & Immunology,** Dalhousie University, Halifax, Nova Scotia, Canada*
*3 - **Department of Biology,** University of New Brunswick, Saint John, New Brunswick, Canada*
*4 - **Department of Chemistry & Biochemistry,** Université de Moncton, Moncton, New Brunswick, Canada*

***Correspondence to Stephen M. Lewis:** Atlantic Cancer Research Institute, Moncton, New Brunswick, Canada.*
stephenl@canceratl.ca
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Abstract

Heterogeneous nuclear ribonucleoprotein A1 (hnRNP A1) is a stress granule-associated RNA-binding protein that plays a role in apoptosis and cellular stress recovery. HnRNP A1 is a major non-histone target of protein arginine methyltransferase 1, which asymmetrically dimethylates hnRNP A1 at several key arginine residues within its arginine–glycine–glycine (RGG)-motif region. Although arginine methylation is known to regulate general RNA binding of hnRNP A1 *in vitro*, the functional role of arginine methylation in hnRNP A1 cytoplasmic activity is unknown. To test the impact of key methylarginine residues on hnRNP A1 cytoplasmic activity and stress granule association, cytoplasmically restricted Flag-tagged mutants of hnRNP A1 were generated in which key methylarginine residues within the RGG-motif region were changed to either lysine or alanine. Lysine substitution, which mimics unmethylated arginine, resulted in a 40% increase in internal ribosome entry site *trans*-acting factor (ITAF) activity and the protein readily associates with stress granules. Alanine substitution resulted in a loss of ITAF activity and reduced mRNA binding. The alanine mutant also displays reduced stress granule association and suppresses stress granule formation. Our data suggest that arginine residues within the RGG-motif region are critical for hnRNP A1 cytoplasmic activities and that endogenous asymmetric dimethylation of the RGG-motif region suppresses hnRNP A1 ITAF activity in cells. Our findings indicate that methylarginine residues within the RGG-motif region of hnRNP A1 are important for its cytoplasmic activities and that hypomethylation and/or mutation of the RGG-motif region may contribute to the role of hnRNP A1 in diseases such as cancer.

Introduction

Post-translational protein arginine methylation regulates the activity and biogenesis of several families of RNA-binding proteins, including the U small nuclear ribonucleoproteins [1], fragile-X mental retardation protein [2], SR splicing factors such as SF2/ASF [3] and the heterogeneous nuclear ribonucleoproteins (hnRNP) [4–6]. Dimethylation of arginine residues can be symmetric, with one methyl group transferred sequentially to each eta (η) nitrogen of a single arginine residue, or asymmetric, with two methyl groups transferred to one η nitrogen. Protein arginine methyltransferase 1 (PRMT1) asymmetrically dimethylates arginine residues in the motif Arg-Gly-X, with X most commonly being glycine or serine [7]. Unlike phosphorylation, arginine methylation is generally non-reversible and there are few examples of functional regulation through dynamic methylation and demethylation. However, several demethylases have been identified that can reverse this post-translational modification, potentially allowing arginine methylation to act as a reversible regulatory signal, such as the histone and estrogen receptor alpha arginine demethylase jumonji domain containing 6 (JMJD6) [8]. Functionally, arginine methylation can regulate protein nuclear export and import and is often associated with nucleo-cytoplasmic shuttling proteins

[3,9]. Protein methylation can also serve as a post-translational signaling modification. For example, lysine methylation can modify the functional activity of p53, depending on which residues are methylated [10]. Finally, arginine methylation of Arg-Gly-Gly (RGG) domains can regulate protein stability, as is the case with the herpes simplex virus 1 protein ICP27 [11].

HnRNP A1 is a nuclear mRNA splicing factor [12–15] and internal ribosome entry site (IRES) *trans*-acting factor (ITAF) [16,17] that is asymmetrically dimethylated by PRMT1 [7]. HnRNP A1 possesses five Arg-Gly-X motifs within its RGG-motif region that are methylated by PRMT1 *in vitro* and *in vivo* [18,19]. Arginine methylation by PRMT1 has been shown to decrease the ability of hnRNP A1 to bind to RNA and single-stranded DNA *in vitro* [19]; however, the precise functional and regulatory role of arginine methylation in hnRNP A1 cellular activities is still unclear. Some studies suggest that arginine methylation regulates nucleo-cytoplasmic shuttling and distribution [3,20], although this is not the case for all hnRNPs. The RGG domain is also known to be involved in protein–protein interactions [21–23]. Arginine methylation of RGG domains does not alter the charge of the arginine residue, but does increase steric hindrance, preventing close interaction of the arginine with the phosphate backbone of single-stranded RNA and therefore may play a role in fine-tuning binding strength of RNA-binding proteins to target mRNA [24].

HnRNP A1 is a nucleo-cytoplasmic shuttling protein [25] that is exported to the cytoplasm as a core member of messenger ribonucleoprotein complexes bound to nascent polymerase II RNA transcripts [25,26]. Following dissociation from the exported complex, hnRNP A1 is reimported into the nucleus through interaction with transportin-1 [27,28]. Phosphorylation of hnRNP A1's M9 non-canonical nuclear localization signal inhibits this interaction and results in cytoplasmic accumulation of hnRNP A1 [29,30]. In the cytoplasm, hnRNP A1 is known to either up- or down-regulate the IRES-dependent translation of various mRNAs that encode proteins related to apoptosis and proliferation, including the X-linked inhibitor of apoptosis (XIAP) [16], c-Myc [31], fibroblastic growth factor 2 (FGF2) [17], apoptotic protease activating factor 1 (Apaf-1) [32], cyclin-D1 [31] and B-cell lymphoma extra-large (Bcl-xL) [33]. HnRNP A1 can also bind the 3′ untranslated region of cellular inhibitor of apoptosis 1 mRNA to decrease its stability [34], demonstrating that regulation of hnRNP A1 activity occurs in part due to its subcellular localization. Dysregulated hnRNP A1 cytoplasmic activity may have an impact on disease states. For example, hnRNP A1 cytoplasmic accumulation occurs in multiple myeloma cells due to aberrant signaling [35,36], which in turn stimulates cell growth through c-Myc overproduction. HnRNP A1 ITAF activity has been specifically targeted to limit c-Myc-mediated cell growth [37], demonstrating its therapeutic potential.

HnRNP A1 is also a known stress granule (SG)-associated protein and plays a role in cellular recovery from stress conditions [38]. SGs are distinct cytoplasmic foci composed of stalled translation machinery and non-translating mRNAs that inhibit viral replication [39] and possibly mediate the sequestration and temporary storage of mRNAs during cellular stress conditions [40]. HnRNP A1 is not required for SG formation but it does interact transiently with SGs [38]. Although nuclear functions and some cytoplasmic functions of hnRNP A1 have been well studied, relatively little is known about the mechanisms that regulate its SG association.

Using an alanine-substitution mutant of hnRNP A1, we demonstrated that key arginine residues within the RGG-motif region of hnRNP A1 are required for mRNA binding, ITAF activity and SG association. Furthermore, using a lysine-substitution mutant, our data suggest that hypomethylation increases hnRNP A1 ITAF activity. Overall, our data suggest that arginine methylation suppresses hnRNP A1 function in the cytoplasm and may serve to finely modulate hnRNP A1 activity.

Results

Asymmetric dimethylation of hnRNP A1 in cells

HnRNP A1 is asymmetrically dimethylated by PRMT1, as demonstrated by mass spectrometry and *in vitro* enzymatic assays [18,19]. We verified the PRMT1-mediated arginine methylation of hnRNP A1 in HeLa whole cell lysates by Western blot analysis using the ASYM25 antibody that recognizes all proteins containing asymmetrically dimethylated arginine residues [41]. HnRNP A1 expression in HeLa cells was decreased by transfection of siRNA that targets hnRNP A1, which resulted in a decrease in the intensity of a 36-kDa protein in the ASYM25 Western blot, suggesting that this protein corresponds to methylated hnRNP A1 (Fig. 1a). We further sought to demonstrate that reduced PRMT1 expression results in reduced hnRNP A1 asymmetric dimethylation in cells. PRMT1 protein expression was reduced by siRNA transfection, which resulted in a reduction in the intensity of the protein species that corresponds to hnRNP A1 in the ASYM25 Western blot (Fig. 1b). In addition, endogenous hnRNP A1 was immunoprecipitated from the cytoplasmic lysates of HeLa cells transfected with PRMT1 siRNA (or a control siRNA) and Western blot was subsequently performed with the ASYM25 antibody to specifically characterize the methylation status of hnRNP A1 (Fig. 1c). Asymmetric dimethylation of hnRNP A1 was significantly decreased in cells with reduced PRMT1 expression, whereas total hnRNP A1 abundance was unchanged. Taken together, these results are consistent with