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Arginine methylation of REF/ALY promotes efficient handover of mRNA to TAP/NXF1

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ABSTRACT

The REF/ALY mRNA export adaptor binds TAP/NXF1 via an arginine-rich region, which overlaps with its RNA-binding domain. When TAP binds a REF:RNA complex, it triggers transfer of the RNA from REF to TAP. Here, we have examined the effects of arginine methylation on the activities of the REF protein in mRNA export. We have mapped the arginine methylation sites of REF using mass spectrometry and find that several arginines within the TAP and RNA binding domains are methylated *in vivo*. However, arginine methylation has no effect on the REF:TAP interaction. Instead, arginine methylation reduces the RNA-binding activity of REF *in vitro* and *in vivo*. The reduced RNA-binding activity of REF in its methylated state is essential for efficient displacement of RNA from REF by TAP *in vivo*. Therefore, arginine methylation fine-tunes the RNA-binding activity of REF such that the RNA–protein interaction can be readily disrupted by export factors further down the pathway.

INTRODUCTION

The transport of mRNA from the nucleus to the cytoplasm is an essential step in eukaryotic gene expression which is coupled with transcription, mRNA processing and translation (1–3). Coupling of these processes ensures efficient loading of export factors and the appropriate release of mRNA for export upon completion of RNA processing. Many of the proteins involved in export are conserved from yeast to humans, including UAP56 (Sub2p), REF/ALY (Yra1p) and TAP/NXF1 (Mex67p) (yeast names in parentheses). REF is initially recruited to sites of transcription through an interaction with Pcf11p, which directly associates with the carboxy terminal domain of the large subunit of RNA polymerase II (Pol II) (4). In addition to the Pcf11-mediated recruitment of REF to sites of transcription, Spt6 binds Pol II and Iws1, which in turn recruits REF to Spt6 responsive genes (5). Subsequently, REF transfers to mRNA and assembles with UAP56 and the THO protein complex to form TREX (6).

REF associates with mRNA through arginine-rich motifs in its N- and C-terminal arms (amino acids 1–73 and 155–218) and RNA also makes weak contacts with loops 1 and 5 of the central RNA recognition motif (amino acids 74–154) (7,8). REF is deposited on mRNA close to the 5′-end and this location is probably governed by the interaction between REF and the CBP80 subunit of the nuclear Cap binding complex (9). UAP56 bridges the interaction between REF and the THO complex (10) and UAP56 together with the THO complex are found further away from the 5′ cap (9). Deposition of REF and TREX on spliced mRNAs depends upon splicing (10,11). However, REF also associates with single exon, unspliced mRNAs in a UAP56-dependent manner (12). UAP56 plays other roles in the cell as it is also directly involved in spliceosome assembly (13) and thus is ideally placed to mediate the coupling between splicing and deposition of REF on spliced mRNAs (14,15). UAP56 (Sub2p) and THO proteins also provide a connection with the 3′-end processing machinery during export and loss of Sub2p/THO leads to polyadenylation defects (16) and the accumulation of an mRNA export intermediate after commitment to 3′-end processing (17). Humans possess a paralogue of UAP56, DDX39 which has overlapping functions with UAP56 and knockdown of both proteins is required to block mRNA export in human cells (18,19).

Once REF is loaded onto mRNA it subsequently recruits TAP, which forms a heterodimer with p15

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(Mtr2p) (20,21). TAP:p15 binding to REF displaces UAP56 and triggers handover of the mRNA from REF to TAP. REF bound to TAP also stimulates the TAP RNA-binding activity (8). ThoC5, a component of TREX, functions as a co-adaptor for mRNA export and binds to TAP together with REF and stimulates the export of specific mRNAs (22). In addition, SR proteins can function as mRNA export adaptors, directly binding TAP in their nuclear hypophosphorylated state (23–25). In common with REF, SR proteins also use an arginine-rich motif adjacent to their RNA recognition motifs to bind TAP (8,26,27). Recently, a further mRNA export adaptor has been identified, UIF, which binds both UAP56 and TAP. UIF is found on the same mRNAs as REF and works together with REF to ensure efficient mRNA export. However, unlike REF, UIF recruitment to mRNA is dependent on the histone chaperone FACT (28). Additional TAP-binding proteins have been identified during a genome-wide screen for mRNA export factors (2), including ZC3H3, which plays a role in coupling 3′-end processing with mRNA export (29). Subsequently, TAP directly associates with nucleoporins and ensures efficient translocation of mRNA across the nuclear pore (30–32). REF is displaced from the mRNA immediately prior to or during its translocation through the nuclear pore (33,34). The displacement of TAP from mRNA in the cytoplasm involves Gle1, Dbp5 and inositol hexakisphosphate (IP_6) (35,36). Dbp5 is an RNA helicase which resides on the cytoplasmic side of the nuclear pore and IP_6 bound Gle1 stimulates the ATPase activity of Dbp5, switching it from the ATP to the ADP bound state. This switch is thought to trigger displacement of TAP from the mRNA (3).

Protein arginine N-methyltransferases (PRMTs) catalyse the post-translational transfer of a methyl group from the donor *S*-adensoyl-L-methioinine (SAM) to arginine residues (37,38). Three forms of arginine methylation have been described N^G-monomethylarginine, N^GN^G-dimethylarginine (asymmetric dimethylarginine aDMA) and $N^GN'^G$ dimethylarginine (symmetric dimethylarginine sDMA). Arginine methylation is a common post-translational modification (PTM) in RNA-binding proteins, including proteins involved in mRNA export (37,39). This PTM has the ability to modify the RNA-binding activity of substrate proteins and influence protein–protein interactions. In the case of HIV-1, the methylation of the viral mRNA export factor Rev leads to a reduced interaction with the Rev response element RNA and reduced viral mRNA export (40). In the case of Npl3, a yeast SR-like protein, arginine methylation reduces its interaction with the Tho2 component of the TREX complex (39). Since a number of arginine residues in REF are associated with binding RNA and TAP, we were interested in investigating how arginine methylation might influence these interactions. Here, we show that whilst arginine methylation of REF does not influence the interaction with TAP, it reduces the interaction with RNA, such that REF can be readily displaced from mRNA by TAP.

MATERIALS AND METHODS

Chemicals

Acetonitrile (LC MS grade), water (HPLC grade), formic acid (FA, HPLC grade) trifluoroacetic acid (TFA, HPLC grade) and ammonium formate (MS grade) were obtained from Fisher Scientific UK.

Plasmids

FLAG-Myc-REF2-I, 13-Myc-TAP, GST-TAP and pET9a/p15 were described in (8). The FLAG-Myc-REF2-I with all 10 methylated arginines mutated to lysine (FLAG-Myc-REF2-I 10R->K) was generated using overlapping PCR primers and the wild type REF2-I open reading frame as a template. Details of oligonucleotides used can be supplied on request.

FLAG-Myc-REF2-1 purification

One 15-cm plate of 293T cells was transfected using calcium phosphate with p3X-FLAG-Myc-REF2-1 and lysed after 48 h in 500 µl lysis buffer (1× PBS, 1% Triton X100) supplemented with 2 mM PMSF (Sigma) and Complete (Roche) protease inhibitors. Cell debris was removed by centrifugation (16.1 rcf, 10 min, 4°C) and the supernatant was incubated for 3 h at 4°C with 30 µl α-FLAG M2-agarose slurry (Sigma) pre-equilibrated overnight in lysis buffer containing 1% BSA. Beads were then washed twice with washing buffer (1× PBS, 1 M NaCl, 0.5% Triton X100) and once with 1× TBS (50 mM Tris pH 7.4, 150 mM NaCl). Purified protein was eluted in 50 µl 1× TBS containing 100 µg/ml 3X-Flag peptide (Sigma) for 30 minutes at 4°C, before analysis by SDS-PAGE stained with Coomassie blue.

GST pull-down assays and Co-IPs using AdOx-treated cell extracts

Transfected 293T cells were cultured for 24 h before 20 µM methylase inhibitor adenosine-2′,3′-dialdehyde (AdOx, Sigma) was added to the medium for an additional 24 h. Pull-down assays were performed in PBS + 0.1% Tween in the presence of 5 µg of RNase as described in (41). Co-IP assays from transfected 293T extracts treated with RNase were performed as described in (26). GST-TAP and p15 were produced by coexpression of pGEX6P1-TAP and pET9a-p15 in *BL21-RP* cells (Novagen).

In vitro cross-linking assays

A 15-mer RNA oligonucleotide (5′-CAGUCGCAUAGU GCA-3′) (Dharmacon) was end-labelled with γ-^{32}P-ATP and 2 µl probe was added to 1 µg purified FLAG-Myc-REF2-I (see previous paragraph) in 20 µl RNA-CL buffer (15 mM HEPES pH 7.9, 75 mM NaCl, 100 µM KCl, 0.2 mM EDTA, 5 mM $MgCl_2$, 0.05% Tween 20, 10% glycerol) for 15 min on ice. Complexes were then UV-irradiated or not on ice before treatment with 1 µl 10 mg/ml RNase A for 15 min at 37°C, and analyzed by SDS-PAGE stained with Coomassie blue followed by PhosphoImaging.

In vivo RNA displacement assay

Transfected 293T cells were UV-irradiated on ice with 0.120 J/cm^2 and lysed in IP lysis buffer (50 mM HEPES pH 7.5, 100 mM NaCl, 1 mM EDTA, 0.5% Triton, 10% glycerol) containing protease inhibitors. Extracts were clarified by centrifugation (16.1 rcf, 5 min, 4°C) and supplemented with 900 mM NaCl before immunoprecipitation using 30 µl α-FLAG M2-agarose slurry (Sigma) for 2 h at 4°C. REF2-I:RNA complexes were eluted in 50 µl IP lysis buffer containing 100 µg/ml 3X-Flag peptide (Sigma) for 30 min at 4°C before treatment with 5 µg of RNaseA for 30 min at 37°C. The remaining RNA bound to REF2-I was end-labelled with γ-^{32}P-ATP in presence of 5 mM $MgCl_2$ and complexes were analysed by western blotting and PhosphoImaging.

mRNP capture assay

PBS-washed transfected 293T cells were UV-irradiated or not on ice with 0.120 J/cm^2 and mRNP capture assays were performed in denaturing conditions as described in (42) except for elution for which washed complexes were directly eluted in 50 µl elution buffer (10 mM Tris pH 7.5, 1 mM EDTA, 0.4 mg/ml RNase A). Captured mRNA-binding protein complexes were analysed by western blotting.

RNA-binding affinity assay

RNA affinities of purified methylated and un-methylated FLAG-Myc-REF2-I were measured by using reactions containing 2.5 µg of immobilized proteins and 0.1875–30 µM 19-mer ^{32}P-end-labelled RNA oligonucleotide (5′-UUGCGCAGUGGAGUUCAAC-3′) in 50 mM NaP (pH 7), 50 mM NaCl, 1 mM $MgCl_2$, 0.1% Tween 20 (Sigma). Beads were washed before Cerenkov counting the bound radioactivity with a Beckman counter.

In vitro methylation reactions

Histidine tagged REF was purified from *Escherichia coli* as described previously (43). GST-PRMT constructs were expressed at 30°C in LB media in 0.1 mM IPTG from pGEX6P and purified using glutathione-Sepharose resin. Enzymes were eluted using 20 mM reduced glutathione then dialysed against 10% glycerol in 50 mM Tris–HCl pH 8.0. Methylation reactions were carried out in 50 mM Tris–HCl, 5 mM $MgCl_2$, 4 mM dithiothreitol pH 9.0 at 30°C with the addition of 320 µM SAM for 2 h.

Protein digestions

Following purification, REF was digested with trypsin (Sigma proteomics grade, 0.1–200 ng) in 100 mM ammonium bicarbonate, 20% acetonitrile at 37°C for 1–6 h. The reactions were quenched by the addition of 0.1% TFA. The samples were subsequently dried under vacuum and re-suspended in 0.1% final concentration of TFA. Six microlitres were used for LC-MS/MS analysis.

ESI MSMS analysis

Peptides were separated using an Ultimate 3000 capillary liquid chromatography system (Dionex UK), using a 75 µm i.d. × 15 cm PepMap reverse phase column (Dionex UK). Linear gradient elution was performed using buffer A (0.1% formic acid) and buffer B (0.1% formic acid, 95% acetonitrile) starting from 5% buffer B to 40% over 40 min at a flow rate of 300 nl/min. Direct injection analysis was performed using Atlantis C18 capillary column 300 µm i.d. × 15 cm (Waters, UK). Linear gradient elution was performed starting at buffer 5% buffer B to 40% buffer B over 40 min at a flow rate of 2 µl/min. Separations at neutral pH were performed using a linear gradient elution; buffer A (20 mM ammonium formate pH 7.0) buffer B (20 mM ammonium formate pH 7.0, 95% acetonitrile) starting at 5% buffer B to 95% buffer B over 40 min at a flow rate of 2 µl/min. MS/MS analysis was performed using a HCT Ultra PTM Discovery instrument [with Esquire control, Data analysis and Biotools for automated data acquisition and processing (Bruker Daltonics, GmbH, Germany) MS1 profile scans (m/z 300–1800] were acquired in standard enhanced positive mode and were followed by two CID and ETD fragmentation experiments in alternating fashion in ultra scan mode (*m*/*z* 100–1800). For fragmentation, the trap was loaded to a target value of 200 000 with a maximum accumulation time of 200 ms. The precursor isolation width was set to 4.0 and singly charged precursors were excluded from MS/MS analysis. For ETD fragmentation, fluoranthene was allowed to accumulate to a target value of 500 000 and the reaction was allowed to proceed for 100 ms. Profile data were then processed into peak list by data analysis using the following settings. The apex peak finder algorithm was used for peak detection using a peak width at half maximum (FWHM) of 0.1 m/z, a signal-to-noise ratio (S/N) of 1.0, a relative to base peak intensity of 0.1% and an absolute intensity threshold of 10. Spectra were deconvoluted with charge state deconvolution from fragment spectra allowed. Peak lists were then exported as Mascot Generic Files (MGF) and searched using Mascot 2.2 server with ETD fragmentation rules specified. Mass accuracies were set to 1.8 Da in MS1 mode and 0.6 Da in MS2 mode. Methionine oxidation, dimethylation and methylation of arginine were used as variable modifications in searches against the Swissprot database (Swiss-Prot Release 51.6, 6 February 2007, 257 964 sequences). ESI TOF MS analysis was performed using a QStar XL, instrument (Applied Biosystems ABI), MS profile data was acquired using Analyst QS 2.0 (Applied Biosystems ABI). Profile data were stored in .wiff format. Peak lists were created using Mascot 1.6b20 script for Sciex Analyst to create MGF and submitted to automated database searching using Mascot (version 2.2 Matrix Science) search engines. IDA survey scan centroids were calculated at 50% peak height and charge states determined. For MS/MS data, centroids were calculated at 50% peak height. Peptides scoring <25 were automatically rejected, whereas all modified peptides scoring >25 were subjected to manual verification with the aid of Biotools within Compass software.

RESULTS

Identification of arginine methylation sites in REF using MS

To identify sites of arginine methylation in REF2-I, hereafter referred to as REF, a FLAG-Myc- tagged REF cDNA expression vector carrying an SV40 origin of replication was transfected in human 293T cells. Western blotting analysis indicated the FLAG-Myc REF was overexpressed ~2.3-fold with respect to endogenous ALY/REF in these experiments (Supplementary Figure S1). FLAG-Myc-REF was immunopurified under stringent conditions with 1M NaCl washes to remove any bound proteins and the resulting purified protein, which appeared as a single band by Coomassie staining (Figure 1A) was digested with trypsin and analysed by MS. Comprehensive analysis was performed using a range of MS approaches, including collision induced dissociation (CID) and electron transfer dissociation (ETD) MS analysis, in conjunction with peptide separations at both neutral and acidic pH (44). The combined MS analysis resulted in the identification of arginine methylation at 10 sites in REF, the detailed tandem MS data for each peptide are shown in Supplementary Figure S2 and summarized in Figure 1B. The MS analysis also identified the presence of both arginine monomethylation and dimethylation. Moreover, a number of arginine residues were identified as either monomethylated or dimethylated, demonstrating the heterogeneous methylation of REF *in vivo*. The MS analysis also enabled us to distinguish the type of arginine dimethylation present. The combined analysis of the characteristic neutral losses and specific precursor ion generated from the tandem MS spectra (45–47) and ETD MS spectra (44) enabled the verification of asymmetric dimethylation in those peptides where arginine dimethylation was identified. The tandem MS spectra of two arginine methylated peptides (N(dimeR)PAIA (dimeR)GGR and S(dimeR)GSGGFGG(dimeR) GSQG(dimeR)G(dimeR)GTGR are shown in Figure 1C and D, respectively. The neutral loss of dimethylamine (−45.06 Da) and specific precursor ions (46.06 Da) are highlighted in the spectra, confirming asymmetric dimethylation in each case.

PRMT1 methylates REF *in vitro*

To further analyse arginine methylation of REF, *in vitro* methylation reactions were performed using a range of protein arginine methyltransferase (PRMTs 1,3,4,5,6) in conjunction with recombinant REF. Following *in vitro* methylation reactions, MS analysis was performed to identify and characterize the sites of arginine methylation of REF *in vitro*. The MS analysis revealed that arginine methylation was only observed following incubation with PRMT1. A number of sites similar to those identified *in vivo* were methylated *in vitro* by PRMT1 (see Supplementary Table S1). The methylation of REF by PRMT1 is also consistent with the identification of asymmetric dimethylation *in vivo* as PRMT1 is a type I methyltransferase which results in asymmetric dimethylation, in contrast to type II methyltransferases such as PRMT5 which results in symmetric methylation (48). PRMT1 was also shown to co-immunoprecipitate with REF in human cells *in vivo* (Figure 2A) and pull-down assays demonstrated an interaction between GST-PRMT1 and REF (Figure 2B). These data are also consistent with the observation that the yeast orthologue of PRMT1, Hmt1, methylates the REF orthologue, Yra1 (39).

Arginine methylation of REF does not affect TAP binding

Both R24 and R32 of REF are involved in binding TAP (8) and were shown to be methylated. Therefore, we investigated whether this post-translational modification influenced the REF:TAP interaction. Extracts were prepared from 293T cells transfected with a FLAG-Myc-REF expression vector, which had been incubated with or without the methylation inhibitor adenosine dialdehyde (AdOx). Analysis of total extracts revealed a clear electrophoretic mobility shift for REF following the inhibition of methylation (Figure 3A). Furthermore, inhibition of methylation was confirmed by MS analysis of immunoprecipitated REF from AdOx-treated cells. Methylation was only observed at three sites compared with ten in the absence of AdOx (Supplementary Table S2). It is highly unlikely that AdOx preferentially blocks methylation of specific arginines within REF, which are normally methylated. Therefore, even those arginines where we can still detect methylation, are likely to have reduced levels overall compared with non-AdOx-treated REF. The qualitative nature of the MS analysis does not allow us to determine the precise level of methylation on individual arginines. However, the MS data are consistent with an overall reduction in the levels of methylated arginines.

We used a pull-down assay to analyse the interaction between GST–TAP and cell extracts containing FLAG-Myc-REF. Analysis of the pull-downs indicated that TAP bound to unmethylated and methylated REF with similar efficiencies (Figure 3A). We further investigated the REF:TAP interaction using co-immunoprecipitation (Co-IP) assays and this analysis showed that Co-IP of FLAG-Myc-REF and TAP-13Myc (Figure 3B) or endogenous TAP (Figure 3C) was not affected by arginine methylation. We conclude that arginine methylation does not influence the interaction between REF and TAP. Since arginine methylation is reported to increase the stability of another RNA-binding protein, HIV-1 Tat (49), we further investigated whether arginine methylation of REF might influence the stability of the protein. However, we found no difference in the stability of REF in the presence of the methylation inhibitor AdOx (data not shown).

Arginine methylation influences the interaction of REF with mRNA

To determine the influence of arginine methylation on the ability of REF to bind RNA we immunopurified FLAG tagged REF from 293T cells in the presence or absence of AdOx under stringent conditions. The purified REF protein was UV cross-linked with radiolabelled RNA to examine its ability to bind RNA *in vitro* (Figure 4A).

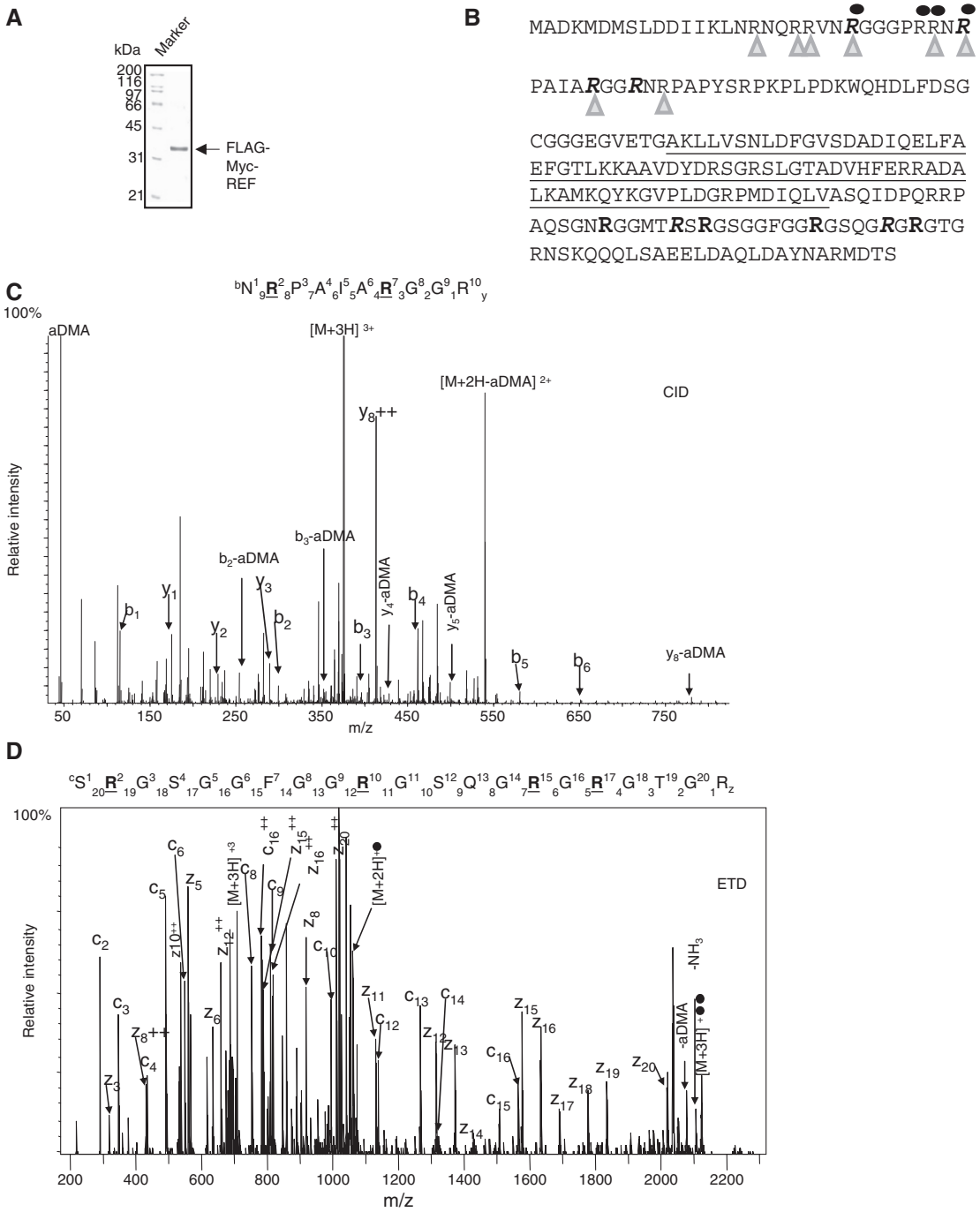

Figure 1. Identification of REF methylation sites. (**A**) Extract from 293T cells transfected with FLAG-Myc tagged REF was subjected to α-FLAG immunopurification in stringent conditions. Purified protein was analysed on SDS-PAGE stained with Coomassie blue. (**B**) Dimethylation (**R**) and both mono or dimethylation (***R***) sites are mapped on the primary sequence of REF together with residues involved in the interaction with TAP (closed circle) and RNA (open triangle). The underlined sequence corresponds to the RRM domain of REF. (**C**) CID MS/MS fragmentation spectra of the methylated peptide N(dmeR)PAIA(dimeR)GGR $[M+2H]^{2+}$. (**D**) ETD MS/MS fragmentation spectra of the methylated peptide S(dimeR)GSGGFGG(dimeR)GSQG(dimeR)G(dimeR)GTGR $[M+3H]^{3}$. The prominent y, b, c′, z′• ions and the characteristic neutral losses/precursor ion associated with asymmetric dimethylation (dimethylamine) are highlighted.

Whilst REF showed a clear UV-dependent cross-link with RNA when methylated, the unmethylated REF protein UV cross-linked with RNA much more efficiently. To confirm that the alterations in the RNA UV-cross-linking activity of REF corresponded to changes in its RNA-binding affinity, we measured the K_D for binding RNA. Immunopurified FLAG-tagged REF was purified and assayed for binding radiolabelled RNA without cross-linking using a pull-down assay (8). The results demonstrated that there was a ∼2-fold increase in binding affinity for RNA when REF was purified from AdOx-treated cells and had reduced arginine methylation (Figure 4B). Since the AdOx treatment of cells does not completely block methylation and methylated arginines

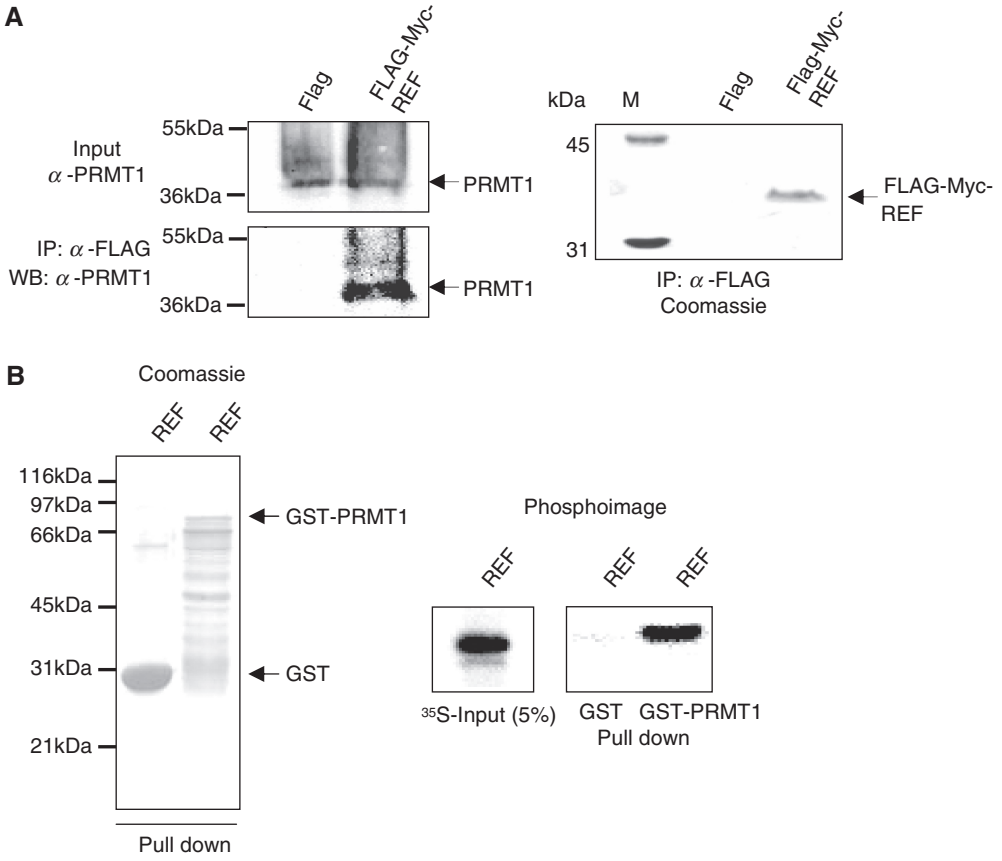

Figure 2. PRMT1 interacts with REF. (**A**) Co-immunoprecipitation of FLAG-Myc-REF with PRMT1. The left two panels show western blots with the PRMT1 antibody. The right panel is a Coomassie stained gel of the immunoprecipitations. FLAG-Myc-REF is expressed at high levels and clearly visible after immunopuriifcation. (**B**) Pull-down assays with GST-PRMT1 and *in vitro* translated REF. The left panel shows the Coomassie stained proteins following the pull-down and the right panels show phosphoimages of the radiolabelled REF input sample and pull-downs.

involved in RNA binding can still be detected (see above and Supplementary Table S2), it is likely that fully demethylated REF would bind mRNA with even greater affinity. To further examine the mRNA-binding activity of REF *in vivo,* we carried out an mRNA capture assay in the presence and absence of AdOx (Figure 4C). This analysis revealed that the presence of AdOx led to significantly more REF protein associated with mRNA *in vivo* than was found in fully methylated non-AdOX-treated cells. The mRNA association was specific since it was abolished when the samples were treated with RNAse and in the absence of UV cross-linking; moreover, FLAG-bacterial alkaline phosphatase (BAP) did not associate with mRNA in this assay. We further examined the influence of arginine methylation on the ability of endogenous human REF/ALY to bind RNA using an mRNP capture assay (Figure 4D). This assay indicated that endogenous REF/ALY is methylated since there was a clear mobility shift when REF/ALY was treated with AdOx (compare lanes 1 and 2). This analysis also revealed that inhibition of arginine methylation led to increased levels of endogenous REF/ALY associated with mRNA *in vivo*. The REF/ALY association with oligodT was specific since it was dependent on UV irradiation, was destroyed by RNAse and no association was detected using IgG Sepharose beads in this assay. We conclude that arginine methylation of REF/ALY reduces its ability to interact with RNA both *in vitro* and *in vivo*.

In vivo role for arginine methylation of REF

During export, mRNA is displaced from REF by TAP, which in turn binds directly to the mRNA. Since arginine methylation influences the RNA-binding activity of REF, we investigated whether this might also influence the handover of mRNA from REF to TAP. We have previously shown that overexpression of TAP *in vivo* leads to a reduction in the steady-state levels of RNA bound to REF (8). Therefore, we carried out the same assay in the presence and absence of AdOX, to examine how arginine methylation would affect the ability of TAP to displace RNA from REF (Figure 5). When REF is methylated, and thus displays reduced RNA UV cross-linking activity, we found as reported previously that TAP could efficiently displace RNA from REF. In contrast, in the presence of AdOx, where REF is unmethylated and shows enhanced RNA UV cross-linking activity, we found that TAP displaced REF from the RNA considerably less efficiently (Figure 5A and B). At the highest concentrations of TAP there was >50%

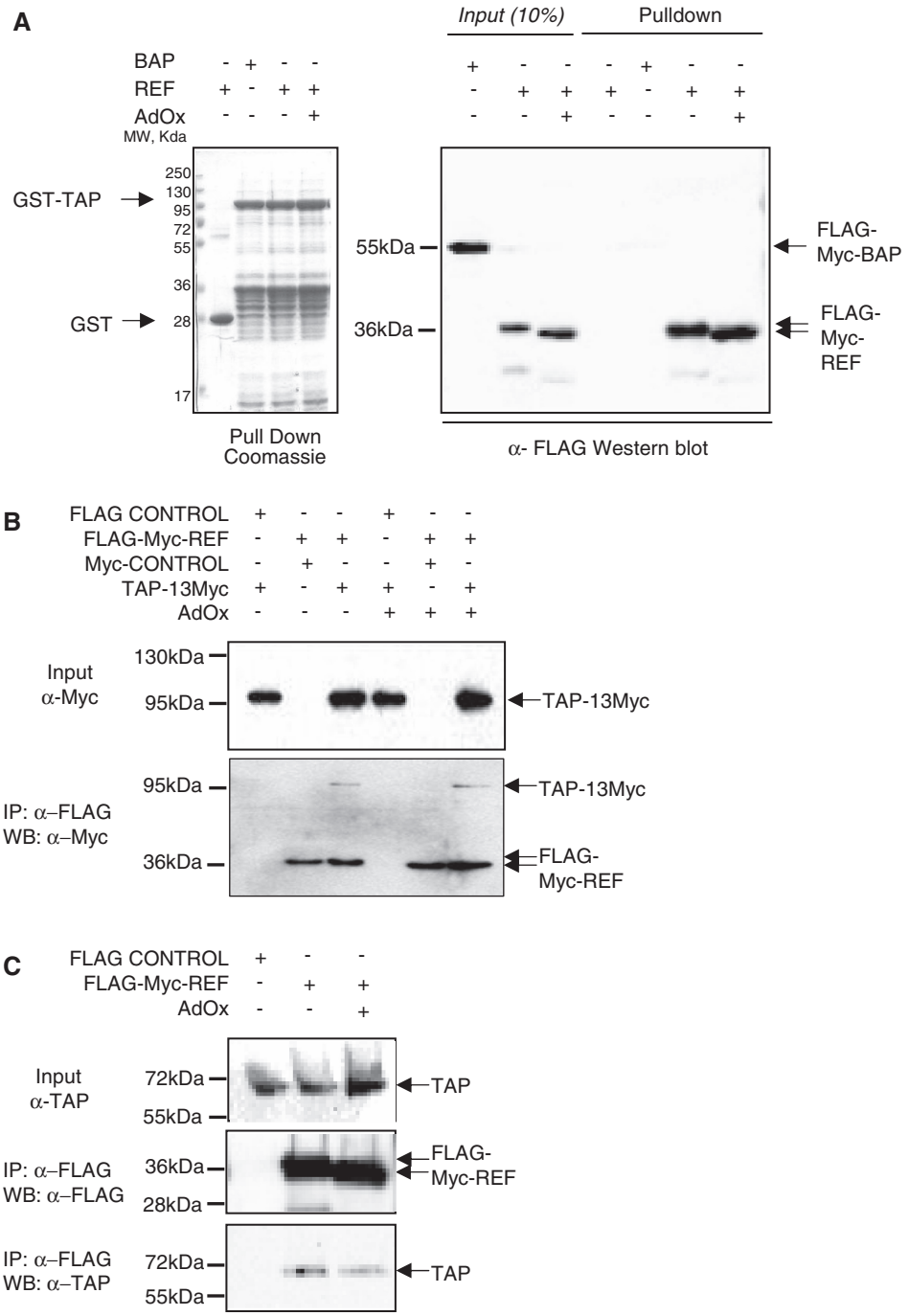

Figure 3. Arginine methylation of REF does not affect the binding of TAP. (**A**) Pull-down assay. GST or GST-TAP-p15 were first immobilized onto glutathione-coated beads before total extracts from +/– AdOx-treated 293T cells transfected with FLAG-Myc-REF were added to the binding reactions. Eluted proteins were analysed by SDS-PAGE stained with Coomassie blue (left) and western blotting (right). A 293T cell extract expressing FLAG-tagged BAP was used as a control (**B**) 293T cells co-transfected with either a FLAG control or a FLAG-Myc-tagged REF and either a 13Myc-tagged TAP or a Myc-control vector were cultured +/– AdOx. Input samples are shown in the top panel following western blotting with α-Myc antibody. Extracts were then subjected to α-FLAG immunoprecipitation (IP) and eluted proteins were analysed by western blotting (WB) with α-Myc antibody (WB) (bottom). (**C**) 293T cells co-transfected with either a FLAG control or a FLAG-Myc-tagged REF were cultured +/– AdOx. Extracts were then subjected to α-FLAG immunoprecipitation (IP) and eluted proteins were analysed by western blotting with α-FLAG (middle) and α-TAP (bottom).

reduction in the RNA cross-linked to REF in the absence of AdOx, whereas in the presence of AdOx, there was only a minor reduction in the amount of RNA cross-linked to REF. We further generated a mutated form of FLAG-Myc-REF in which all methylated arginines were mutated to lysine to examine whether this mutant might be resistant to RNA displacement by TAP, similar to the unmethylated form of FLAG-Myc-REF. Whilst the lysine

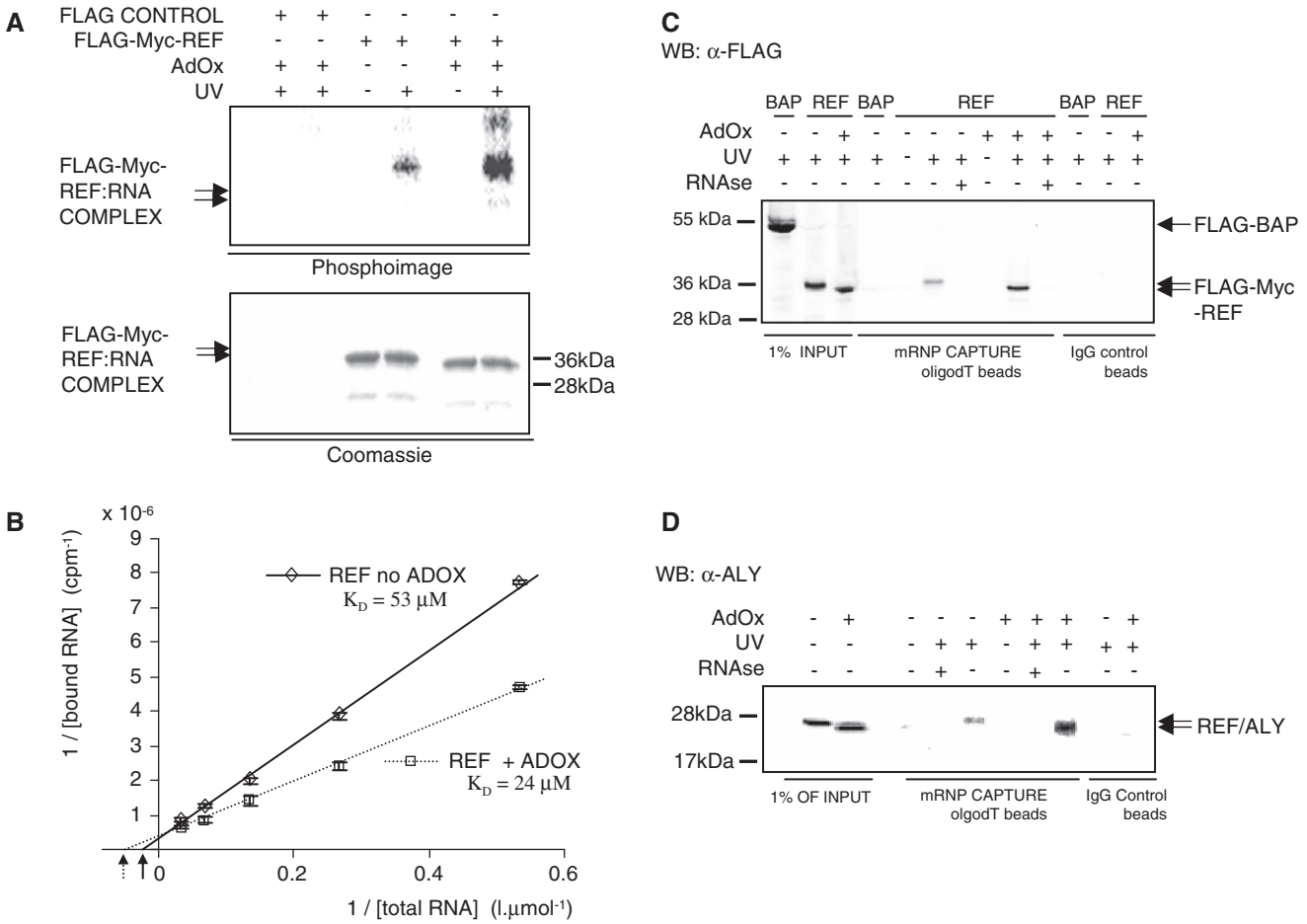

Figure 4. Arginine methylation decreases the mRNA-binding activity of REF and human REF/ALY. (**A**) *In vitro* protein:RNA UV cross-linking assay. Immuno-purified FLAG or FLAG-Myc-REF from transfected 293T cells treated (+) or not (–) with AdOx was UV-cross linked (+) or not (–) with a ^{32}P-radiolabelled RNA oligonucleotide. Resulting complexes were analysed by SDS-PAGE stained with Coomassie blue (lower panel) and Phosphoimage (Upper panel). (**B**) RNA-binding affinities were measured for immunopurified FLAG-REF from ADOX-treated or untreated 293T cells and a ^{32}P-labelled 21-mer oligoribonucleotide as detailed in 'Materials and Methods' section. Standard error bars were calculated from two independent experiments. (**C**) mRNP capture assay. Poly(A)+ RNA from 293T cells +/– AdOx transfected with FLAG, FLAG-Myc-REF (REF) or FLAG-Myc-BAP was purified on oligo-dT or IgG Sepharose beads as indicated in denaturing conditions after UV cross-linking (+) or not (–). Total extract (1% of input) and eluted proteins were analysed by western blotting (WB) with α-FLAG antibody. (**D**) Same experiment as in (C) using untransfected 293T cells and α-ALY antibody to detect the endogenous REF/ALY protein.

mutant form of FLAG-Myc-REF still bound TAP well, it showed very weak RNA-binding activity compared with wild-type FLAG-Myc-REF (Supplementary Figure S3A and B) and we were unable to carry out the displacement assay with TAP. We conclude that arginine methylation of REF leads to reduced RNA-binding activity, which allows TAP to displace the RNA bound to REF. When arginine methylation is inhibited, the affinity for RNA increases, preventing its efficient displacement from REF by TAP.

DISCUSSION

We have shown that arginine methylation leads to substantially reduced RNA-binding activity for REF and decreased association with mRNA *in vivo*. We previously identified arginine residues within REF associated with mRNA binding using NMR and biochemical techniques (7) and have now shown that three of the arginine residues previously implicated in RNA binding are subject to methylation (Figure 1). The earlier studies on REF RNA binding did not indicate which residues within the REF C-terminal arginine-rich RNA-binding domain were specifically involved in RNA binding. However, a number of arginine methylation sites within the C-terminal RNA binding domain (amino acids 153–198) have been identified in this study, raising the possibility that further arginines involved in RNA binding are methylated. The asymmetric dimethylation of arginine will lead to the loss of two potential hydrogen bond donors on the methylated guanidino nitrogen, which may otherwise be involved in RNA binding. Furthermore, the modification will result in the addition of bulky methyl groups, which could sterically hinder protein–RNA interactions. In the case of REF, it is clear that these post-translational modifications have the effect of reducing its RNA-binding activity.

Diminished RNA-binding activity following arginine methylation has been reported previously for HIV-1 Rev

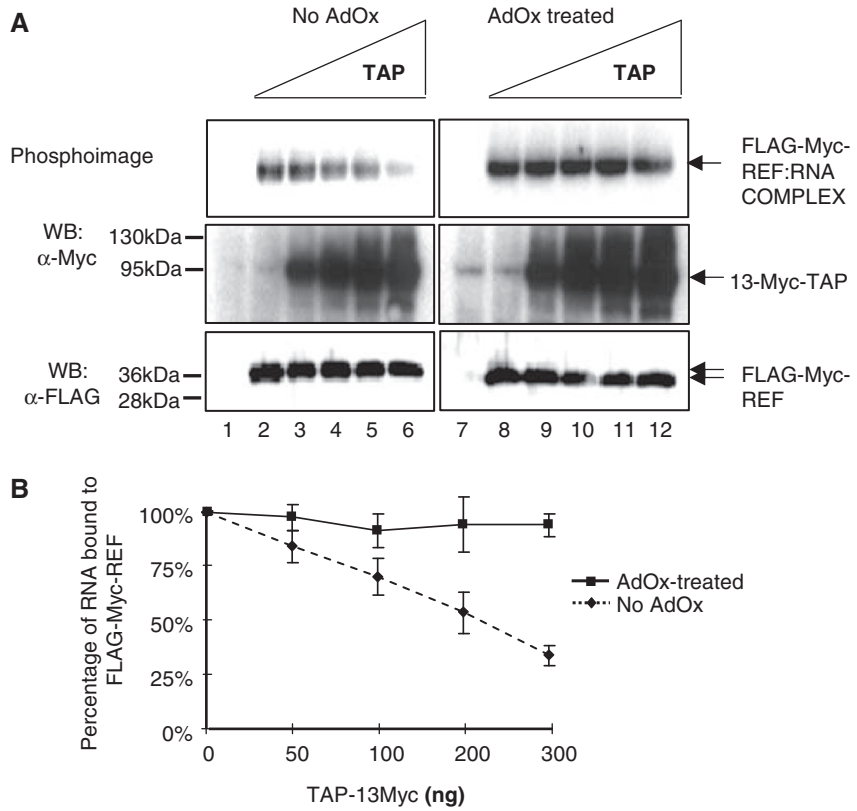

Figure 5. Arginine methylation of REF facilitates the RNA handover from REF to TAP-p15 during mRNA export. (**A**) *In vivo* competition assay. 293T cells transfected with either a FLAG control (lanes 1 and 7) or FLAG-Myc-REF (lanes 2–6 and 8–12) and increasing amounts of 13-Myc-TAP and p15 expression plasmids, were treated +/– AdOx before UV cross-linking. Extracts were subjected to α-FLAG immunoprecipitation and REF:RNA complexes were treated with RNase. End-restricted mRNAs cross-linked to REF were radiolabelled using polynucleotide kinase before phosphoimaging (Top). The increasing expression of TAP was confirmed in total extracts by α-Myc western blotting (WB) (Middle) and specific immunoprecipitation of FLAG-Myc-REF was confirmed by α-FLAG WB (Bottom). (**B**) Quantification of the radiolabelled RNA detected on the phosphoimage of the competition assay. Error bars represent the standard error of the mean from three independent experiments.

protein (40). In this case, mutagenesis studies indicated that there were three potential arginine methylation sites within the RNA-binding domain, with the MS data suggesting that in fact only one of these three potential sites was methylated, although which one was not defined. A more recent systematic study of the effects of asymmetric arginine dimethylation of the RNA-binding peptide from Rev and its interaction with the Rev response element (RRE) RNA has shown that out of 10 potential sites for arginine dimethylation, methylation of eight of them led to a reduced binding affinity for the Rev peptide: RRE interaction, with decreases in binding affinity ranging from 1.4- to 25-fold (50). Interestingly, asymmetric dimethylation of two arginines led to modest increases in the Rev–RRE interaction and it was suggested that the small increases might have resulted from new hydrophobic interactions. Thus, which specific arginine residues are methylated can have a significant bearing on the interaction between an RNA-binding protein and RNA.

The reduced RNA-binding affinity observed for REF following arginine methylation ensures the RNA can be efficiently displaced by TAP during mRNA export (Figure 5). This handover process is unlikely to be unique to the REF:TAP interaction and, in fact, many of the proteins involved in RNA processing interact transiently with their RNA substrates. Moreover, many of these proteins, including hnRNP proteins (51) and Y14 (52), an exon junction complex component, are arginine methylated. For a number of these proteins, arginine methylation may be required to ensure they bind RNA with a suitable affinity to allow reversal of the protein:RNA complex interaction as RNA processing proceeds. As such arginine methylation may be used to fine tune protein:RNA interactions, in the same way that it is involved in the regulation of protein:protein interactions.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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