Heterogeneous nuclear ribonucleoprotein E1B-AP5 is methylated in its Arg-Gly-Gly (RGG) box and interacts with human arginine methyltransferase HRMT1L1

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The heterogeneous nuclear ribonucleoprotein (hnRNP) family includes predominantly nuclear proteins acting at different stages of mRNA metabolism. A characteristic feature of hnRNPs is to undergo post-translational asymmetric arginine methylation catalysed by different type 1 protein arginine methyltransferases (PRMTs). A novel mammalian hnRNP, E1B-AP5, recently identified by its interaction with adenovirus early protein E1B-55 kDa, has been proposed to have a regulatory role in adenoviral and host-cell mRNA processing/nuclear export [Gabler, Schutt, Groitl, Wolf, Shenk and Dobner (1998) J. Virol. **72**, 7960–7971]. Here we report that E1B-AP5 is methylated *in vivo* in its Arg-Gly-Gly (RGG)-box domain, known to mediate protein–RNA interactions. The activity responsible for E1B-AP5 methylation forms a complex with E1B-AP5 *in vivo*. The predominant mammalian arginine methyltransferase HRMT1L2 (hPRMT1) did not detectably methylate endogenous E1B-AP5 despite efficiently methylating a recombinant RGG-box domain of E1B-AP5. Using yeast two-hybrid screening we identified HRMT1L1 (PRMT2) as one of the proteins interacting with E1B-AP5. By *in situ* immunofluorescence we demonstrated that E1B-AP5 co-localizes with the nuclear fraction of HRMT1L1. The Src homology 3 (SH3) domain of HRMT1L1 was essential for its interaction with E1B-AP5 *in vivo*. We suggest that HRMT1L1 is responsible for specific E1B-AP5 methylation *in vivo*.

Key words: periodate-oxidized adenosine, *S*-adenosylmethionine, SH3 domain.

INTRODUCTION

Heterogeneous nuclear ribonucleoproteins (hnRNPs) are nuclear or nuclear–cytoplasmic shuttling proteins that interact avidly with themselves and form complexes with polymerase II transcripts. Their diverse functions cover the regulation of all steps of mRNA metabolism in the cell, ranging from transcription and pre-mRNA processing in the nucleus to cytoplasmic mRNA translation and turnover (reviewed in [1–3]). A recently identified new member of the hnRNP family, E1B-AP5, interacts with adenoviral early protein E1B-55 kDa [4]. Selective accumulation of adenovirus mRNA species is dependent on early adenovirus E1B-55 kDa protein and the same regions of E1B-55 kDa that mediate binding to E1B-AP5 are crucial for normal virus growth [4]. E1B-AP5 overexpression stimulates the export of adenoviral mRNA transcripts and simultaneously prevents the block of host-cell mRNA export in lytically infected cells, indicating that E1B-AP5 is involved in both viral and cellular mRNA metabolism [4]. E1B-AP5 also binds to the N-terminal domain of TAP, an essential RNA export mediator that might bridge the interaction between specific RNP export substrates and the nuclear pore complexes [5]. E1B-AP5 is highly similar to the multifunctional protein hnRNP-U/SAFA, which was shown to be a scaffold attachment factor [6,7] and an inhibitor of polymerase II elongation [8].

Many of the proteins involved in RNA metabolism contain regions with clustered arginine residues in an Arg-Gly-Gly (RGG) motif (RGG box), which might act in protein–RNA interactions by destabilizing the secondary structure of RNA [9,10] and in combination with other RNA-binding domains increase the affinity of a protein for RNA [11–14]. A unique feature of RGG regions is that arginine residues in this context undergo post-translational asymmetric dimethylation catalysed by type 1 protein arginine methyltransferases (PRMTs) (reviewed in [15]). hnRNPs comprise a major group of substrates for this class of enzymes, which catalyse the formation of asymmetric ω-N^G,N^G-dimethylarginine residues present in the RGG or Arg-Xaa-Arg sequence motifs. In mammalian cells hnRNPs contain approx. 65% of the total N^G,N^G-dimethylarginine found in the cell nucleus. Additionally, functionally diverse proteins are substrates for PRMT family members, including fibrillarin and nucleolin, involved in ribosomal biogenesis [15], histones [15,16], fibroblast growth factor [17], interleukin enhancer-binding factor 3 [18], the SRC kinase adapter protein Sam68 [19], STAT1 (signal transducer and activator of transcription 1) [20] and ewing sarcoma protein [21]. The wide range of substrates accounts for the diverse functional roles of asymmetric protein arginine methylation, which facilitates nuclear hnRNP export in yeast [22] and is involved in subcellular protein localization [17], ligand-induced signal transduction [18], transcriptional and post-transcriptional regulation [16,20,23,24] and the modulation of specific protein–protein interactions [19] in mammalian cells. This post-translational modification is also thought to contribute to interactions between protein and nucleic acid [15,25].

Although protein arginine methylation has been known for more than 30 years, it was only recently that several PRMT genes

Abbreviations used: AdOx, periodate-oxidized adenosine; GAR, glycine–arginine-rich; GFP, green fluorescent protein; GST, glutathione S-transferase; HA, haemagglutinin A; hnRNP, heterogeneous nuclear ribonucleoprotein; Hmt, hnRNP methyltransferase; mAb, monoclonal antibody; PRMT, protein arginine methyltransferase; SAM, *S*-adenosylmethionine.

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were cloned and enzymic activity was characterized for most of their products. Several mammalian PRMTs with partly overlapping substrate specificity *in vitro* have been described: PRMT1 from rat and the human homologue HRMT1L2 [26–28], human PRMT2 (HRMT1L1) [27], rat and human PRMT3 [29], mouse CARM1 (PRMT4) [16] and human JBP1 (PRMT5) [30]. The differential substrate specificities *in vivo* of the different mammalian PRMTs remain unclear.

Human HRMT1L1 and HRMT1L2 cDNA species were identified by expressed sequence tag cloning on the basis of their similarity to yeast hnRNP methyltransferase Hmt1 [27]. HRMT1L2, a true homologue of a predominant rat methyltransferase PRMT1 [18], was shown to methylate human hnRNPA1, yeast Np13 and fibroblast growth factor *in vitro* and to complement Hmt1 deficiency in yeast [27,31]. HRMT1L1 is the only PRMT family member whose activity has not yet been demonstrated. On the recognition that HRMT1L1 is the only methyltransferase containing the Src homology 3 (SH3) domain, which is known to mediate a broad range of regulatory protein–protein interactions (reviewed in [32–34]), a protein cofactor binding requirement for HRMT1L1 activity was proposed [27].

Here we show that the novel hnRNP family member E1B-AP5 is methylated *in vivo* and *in vitro* in its RGG-box domain, which is involved in mediating protein–RNA interactions. The methyltransferase activity was co-immunoprecipitated with E1B-AP5 out of human cell lines and was responsible for the methylation of E1B-AP5 *in vitro* as well as that of several co-precipitated proteins. Although HRMT1L2 efficiently methylated a recombinant RGG-box domain of E1B-AP5, it did not contribute to endogenous E1B-AP5 methylation. We hypothesized that methyltransferase activity, other than that of HRMT1L2, exists in a complex with E1B-AP5 *in vivo*. Using the two-hybrid screen we identified HRMT1L1 as a protein interacting with E1B-AP5. Both proteins co-localize in the nucleus and their complex formation *in vivo* is mediated by the SH3 domain of HRMT1L1. We suggest that HRMT1L1 is responsible for specific E1B-AP5 methylation *in vivo*.

EXPERIMENTAL

Cell culture and antibodies

H1299 cells were cultured in monolayers in Dulbecco's modified Eagle's medium (Gibco) supplemented with penicillin, streptomycin and 10% (v/v) fetal calf serum (Gibco). The H12-AP5/7 cell line stably expressing haemagglutinin A (HA)-tagged E1B-AP5 was described previously [4] and was propagated under the same conditions in the presence of 0.5 μg/ml puromycin.

Insect *Spodoptera frugiperda* (Sf9) cells were propagated in TC100 medium supplemented with penicillin, streptomycin and 10% (v/v) fetal calf serum at 27 °C.

To obtain hypomethylated cell lysates, cells were grown for 48 h in the presence of the methylation inhibitor periodate-oxidized adenosine (AdOx; Sigma) at a final concentration of 20 μM in the cell medium.

Anti-(E1B-AP5) rabbit polyclonal antibody was generated against bacterially purified E1B-AP5 as described [4]. The same antigen was used to generate rat monoclonal antibodies (mAbs). The specificity of rat antibodies was analysed by the immunoprecipitation of *in vitro* translated antigen with a Protein G–antibody complex as described [4]. Rat mAbs 4A11 and 6C5 as well as rabbit polyclonal anti-(E1B-AP5) antibody specifically recognized E1B-AP5 but not the highly similar hnRNP-U protein (results not shown).

The following commercial antibodies were used in the present study: anti-HA mouse mAb 12CA5 (Roche); anti-FLAG M2 (where the FLAG epitope is Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys) mouse mAb (Sigma); peroxidase-conjugated anti-mouse, anti-rat and anti-rabbit antibodies (Pharmacia/Amersham); FITC-conjugated anti-mouse antibody (Alexa), Texas Red (Dianova).

Plasmids

pHA–E1B-AP5wt was constructed by the insertion of HA-tagged full-length E1B-AP5 cDNA from pSVfluE1B-AP5 [4] into *Eco*R1/*Xho*I sites in the pcDNA3 vector (Invitrogen). pHA–E1B-AP5-N was generated by PCR amplification of an HA-tagged N-terminal fragment covering residues 1–454 of E1B-AP5 and cloning the product into the *Eco*R1/*Xho*I sites of pcDNA3. pHA–E1B-AP5ΔRGG was constructed by the deletion of E1B-AP5 residues 612–685 with PCR splicing: fragments corresponding to HA fused to residues 1–611 and residues 686–869 were PCR amplified with partly overlapping 3′ and 5′ primers for the N-terminal and C-terminal fragments respectively, then mixed and used as a template for second-round PCR. The resulting product representing HA-tagged E1B-AP5 lacking the RGG-box domain was cloned in the *Eco*R1/*Xho*I sites of pcDNA3. A description of the recombinant pcDNA3 constructs expressing FLAG-tagged HRMT1L1 full-length and HRMT1L1ΔSH3 (deletion of residues 1–103) is available from the authors on request.

The plasmids for bacterial expression of human methyltransferases pGEX-HRMT1L1 and pGEX-HRMT1L2 [27] were a gift from Michael F. Henry.

The bacterial expression vector pGEX–GFP encoding glutathione S-transferase (GST) linked to green fluorescent protein (GFP) was described previously [35]. To generate plasmids pGST–RGG–GFP and pGST–N/RG–GFP, E1B-AP5-coding regions for residues 611–688 and 171–193 were amplified by PCR with E1B-AP5-specific primers containing *Bam*HI and *Nhe*I restriction sites and with pcDNA3 HA–E1B-AP5wt as a template. The PCR products were digested with *Bam*HI and *Nhe*I and inserted into the vector pGEX–GFP.

Protein purification from *Escherichia coli*

GST-fused HRMT1L1 and HRMT1L2 were expressed in *E. coli* strain BL21-CodonPlus-RIL (Stratagene). Recombinant GST–RGG–GFP, GST–N/RG–GFP and control GST–GFP proteins used as substrates for methylation assays *in vitro* were expressed in *E. coli* strain DH5α. Protein expression and purification were performed under non-denaturing conditions as described [35]. Proteins were eluted from GST–Sepharose in the presence of 15 mM glutathione, dialysed overnight at 4 °C against PBS and stored in aliquots at −80 °C.

Protein concentration determination

Protein concentrations of H1299 and Sf9 cell lysates used for immunoprecipitation, Western blotting and methylation assays *in vitro* were determined in accordance with the Bio-Rad protein assay commercial protocol with BSA dilutions as standards. Amounts of GST-purified proteins and immunoprecipitated proteins were analysed by comparison with BSA standards with the use of gel electrophoresis followed by protein staining with Gelcode Blue stain reagent (Pierce).

Methylation assay *in vivo*

Cell labelling for the analysis of E1B-AP5 methylation *in vivo* was basically performed as described [36], with minor modifications. H1299 cells and H12-AP5/7 were propagated until the

cell density reached 70 %. The medium was replaced with Eagle's minimal essential medium without methionine (Gibco BRL), supplemented with penicillin, streptomycin and 5 % (v/v) dialysed fetal calf serum for 30 min. Protein translation was then inhibited by adding a cycloheximide/chloramphenicol mixture to final concentrations of 100 μg/ml and 40 μg/ml (no-translation mix) respectively to the medium. Cells were incubated with translation inhibitors for 1 h before labelling as well as during the labelling procedure. To detect methylated proteins the methyl-group donor L-[*methyl*-^{3}H]methionine (Amersham) was added to cell medium at a final concentration of 30 μCi/ml. Protein translation inhibition was monitored by cell labelling with 20 μCi/ml [^{35}S]methionine (ICN).

After 3.5 h of metabolic labelling, cells were washed twice with PBS, collected and lysed in NP40-1 % lysis buffer [50 mM Tris/HCl (pH 8.0)/150 mM NaCl/1 % (v/v) Nonidet P40] supplemented with a protease inhibitor cocktail tablet (Roche). Cell lysates were precleared with Protein G–Sepharose. Precleared lysates (200 μg) were incubated overnight with Protein G coupled to the corresponding antibody in total volume of 1.5 ml, then washed five times with NP40-1 % buffer. SDS loading buffer (60 μl) was added to each sample. Samples were heated for 95 °C for 5 min and analysed by SDS/PAGE. Gels were incubated for 1 h with Enlight solution (EnerGene), dried and exposed to Kodak X-OMAT (AR) film at −80 °C.

To analyse the methylation of E1B-AP5 mutants *in vivo*, H1299 cells were transiently transfected with the appropriate pcDNA3-HA–E1B-AP5 constructs by the calcium phosphate precipitation method. Methylation *in vivo* was performed 48 h after transfection. The amount of immunoprecipitated protein was checked by Western blot analysis with anti-(E1B-AP5) rabbit polyclonal antibody, anti-(E1B-AP5) rat mAb 4A11 or 6C5 for the endogenous protein and 12CA5 for the HA-tagged E1B-AP5 expression constructs.

Methylation assay *in vitro*

Cells grown in the presence or absence of AdOx were washed twice with PBS and lysed in the lysis buffers with different stringencies. The following buffers were used: NP40-1 % buffer, RIPA-L buffer [50 mM Tris/HCl (pH 8.0)/150 mM NaCl/1 % (v/v) Nonidet P40/0.1 % (v/v) Triton X-100], RIPA-S buffer [50 mM Tris/HCl (pH 8.0)/150 mM NaCl/1 % (v/v) Nonidet P40/0.1 % SDS/0.5 % sodium deoxycholate] supplemented with protease inhibitor cocktail.

The methylation assay *in vitro* was performed with *in vitro* methylation buffer containing 50 mM Na/Mops, 0.3 M NaCl and 2 mM EDTA supplemented with a protease inhibitor cocktail tablet (Roche). Total reaction volumes, concentrations and sources of methyltransferase activity and substrates are indicated in the Figure legends.

S-Adenosyl[*methyl*-^{3}H]-L-methionine (SAM) obtained from Pharmacia/Amersham was used as a donor of methyl groups; 4 μCi of SAM (specific radioactivity 15 Ci/mmol) was used per 40 μl of reaction mix. Samples were incubated for 40 min at 37 °C and the reaction was stopped by adding 2 × SDS or 5 × SDS sample buffer and heating for 5 min at 95 °C. Aliquots of reaction solution were analysed by SDS/PAGE followed by incubation with Enlight solution (EnerGene) for 1 h, drying and autoradiography at −80 °C. Western blotting was performed in parallel to determine the amount of E1B-AP5 in the reaction.

Yeast two-hybrid screening

Yeast two-hybrid analysis was performed in accordance with standard protocols [37]. The central part of E1B-AP5 corresponding to residues 212–732 was used as bait. The yeast strain Y190 containing pAS E1B-AP5/5 was transformed with a HeLa cDNA library in the pACT vector (S. Elledge). Sixteen clones were identified that interacted with E1B-AP5 in both histidine and β-galactosidase assays. A similarity search with the BLAST algorithm revealed that one of these clones contained a cDNA fragment with an open reading frame corresponding to residues 1–97 from the predicted protein sequence of human arginine methyltransferase HRMT1L1 [27].

Protein–protein interaction analysis with the baculovirus expression system

Recombinant baculoviruses expressing full-length HA-tagged E1B-AP5, FLAG-tagged full-length HRMT1L1 and HRMT1L1 with a deleted SH3 domain were generated with the Bac-to-Bac baculovirus expression system (Gibco BRL) in accordance with the manufacturer's instructions. Sf9 cells were infected by incubation with TC100 medium containing the appropriate combination of recombinant baculoviruses. After incubation for 1 h, virus-containing medium was replaced by TC100 medium supplemented with antibiotics and fetal calf serum. Infected cells were propagated for 48 h, collected and lysed in NP40-1 % lysis buffer. The level of protein expression was monitored by Western blotting with an anti-FLAG mAb for HRMT1L1 constructs and rabbit polyclonal serum or 12CA5 antibody for E1B-AP5. For each immunoprecipitation 50 μg of cell lysate was incubated with the appropriate Protein A-coupled antibody in a total volume of 1 ml for 3 h, then washed five times in the same buffer. After the addition of SDS gel loading buffer, samples were heated for 5 min at 95 °C and analysed by Western blotting as indicated in the Figure legends.

Indirect immunofluorescence

HeLa cells were grown on coverslips, transiently transfected with FLAG-tagged HRMT1L1 by precipitation with calcium phosphate and analysed by indirect immunofluorescence 24 h after transfection. All procedures were performed as described previously [4]. Antibodies were used in the following dilutions: 1:2 for anti-(E1B-AP5) rat hybridoma supernatant, 3.5 μg/ml for anti-FLAG M2, 1:3000 for FITC-labelled anti-mouse, and 1:100 for Texas-Red-conjugated anti-rat. Samples were viewed with a Leica Microscope DM RX HC (Mikrovid). Magnification was × 2000. Pictures were acquired with a SPOT RT slider camera (Diagnostic Instruments).

RESULTS

E1B-AP5 is methylated *in vivo*

Not only does E1B-AP5 share significant similarity with hnRNP U, both proteins migrate with a molecular mass of approx. 120 kDa under PAGE. Therefore, to distinguish between E1B-AP5 and hnRNP U, we combined a methylation assay *in vivo* with immunoprecipitation of E1B-AP5 with 4A11 rat mAb, which specifically recognizes E1B-AP5 translated *in vitro* but not hnRNP U (results not shown). Labelling H1299 cells with L-[*methyl*-^{3}H]methionine in the presence of protein translation inhibitors, followed by immunoprecipitation with the mAb 4A11, revealed the 120 kDa labelled E1B-AP5 protein in SDS/PAGE (Figure 1A, lane 4). Inhibition of protein translation was confirmed by parallel cell labelling with [^{35}S]methionine under the same conditions (Figure 1A, lane 2). Because residual translational incorporation of L-[*methyl*-^{3}H]methionine could be excluded, labelling was due to post-translational methylation.

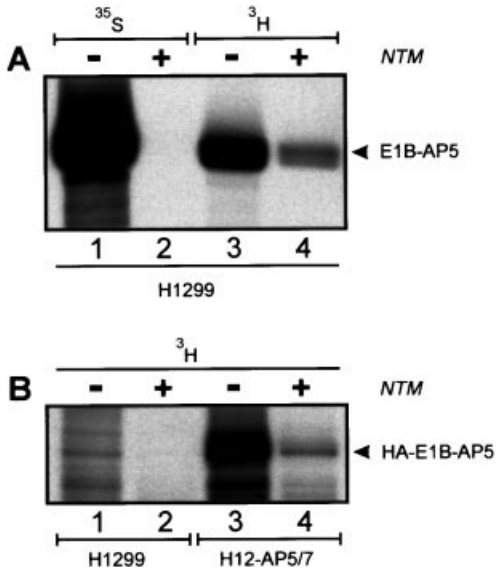

Figure 1 E1B-AP5 methylation *in vivo*

(**A**) Methylation of endogenous E1B-AP5 *in vivo* in H1299 cells. Cells were incubated with medium lacking methionine and labelled with L-[*methyl*-^{3}H]methionine in the presence (lane 4) or absence (lane 3) of no-translation mix (NTM). Protein synthesis inhibition was confirmed by parallel cell labelling with [^{35}S]methionine in the presence (lane 2) or absence (lane 1) of NTM. Immunoprecipitation was with Protein G-coupled anti-(E1B-AP5) 4A11 mAb. Samples were analysed by SDS/PAGE followed by autoradiography and by Western blotting to confirm equal amounts of immunoprecipitated E1B-AP5 in each sample (results not shown). A 5-day exposure is shown. Methylated E1B-AP5 is indicated by the arrow (lane 4). (**B**) Stably expressed HA-tagged E1B-AP5 methylated *in vivo* in H12-AP5/7 cells. H12-AP5/7 (lanes 3 and 4) and control H1299 (lanes 1 and 2) cells were metabolically labelled with L-[*methyl*-^{3}H]methionine in the presence (lanes 2 and 4) or absence of NTM (lanes 1 and 3). Control [^{35}S]methionine labelling (results not shown) and other procedures were performed as above, except that anti-HA mAb was used for immunoprecipitation. HA-tagged methylated E1B-AP5 is indicated by the arrow (lane 4).

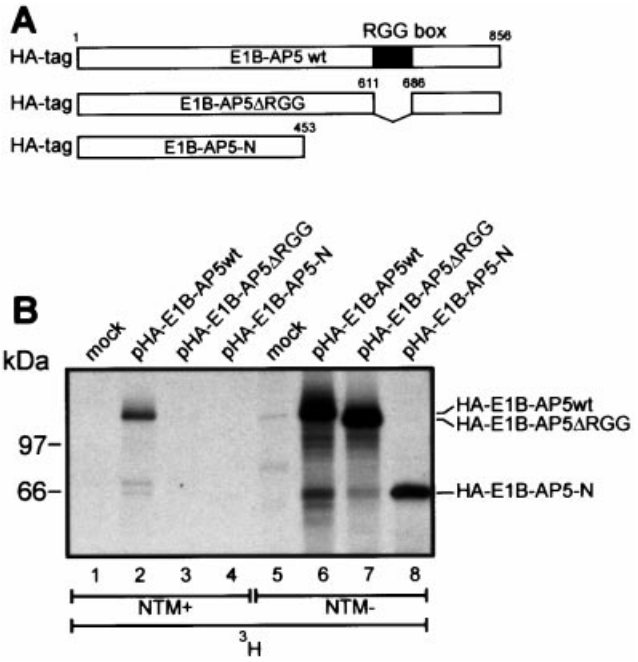

Figure 2 E1B-AP5 is methylated *in vivo* in its RGG-box domain

(**A**) Schematic map of E1B-AP5 constructs used for transient transfection. (**B**) H1299 cells were mock-transfected (lanes 1 and 5) or transiently transfected with 5 μg of HA–E1B-AP5wt (lanes 2 and 6), HA–E1B-AP5ΔRGG (lanes 3 and 7) and HA–E1B-AP5-N (lanes 4 and 8) by precipitation with calcium phosphate. Methylation was performed *in vivo* 48 h after transfection. No-translation mix (NTM) was added in lanes 1–4. Anti-HA mouse mAb was used for immunoprecipitation. Inhibition of protein synthesis was monitored by parallel cell labelling with [^{35}S]methionine. Equivalent levels of HA–E1B-AP5 construct expression and immunoprecipitation were confirmed by Western blotting (results not shown). An 8-day exposure is shown. A methylation signal is observed only for HA–E1B-AP5wt (lane 2), not for HA–E1B-AP5ΔRGG (lane 3) or HA–E1B-AP5-N (lane 4). The positions of molecular mass markers are indicated at the left.

Methylation of endogenous E1B-AP5 was similarly observed in HeLa and MCF7 cells (results not shown). Specific E1B-AP5 methylation was additionally confirmed in a H12-AP5/7 stably expressing full-length HA-tagged E1B-AP5wt by methylation/immunoprecipitation experiments *in vivo* with the anti-(HA-tag) 12CA5 mAb (Figure 1B, lane 4).

E1B-AP5 is methylated in the RGG-box domain

E1B-AP5 contains an RGG-reach region, known to be a substrate for post-translational asymmetric dimethylation catalysed by type 1 arginine methyltransferases, located between residues 612 and 685. To determine whether this region is responsible for E1B-AP5 methylation, we generated mutants lacking the RGG-box domain: E1B-AP5ΔRGG (deletion of residues 612–685) and E1B-AP5-N containing the N-terminal part of E1B-AP5 (residues 1–453) (Figure 2A). HA-tagged full-length E1B-AP5wt, E1B-AP5ΔRGG and E1B-AP5-N were transiently transfected in H1299 cells. At 48 h after transfection, methylation/immunoprecipitation experiments were performed *in vivo* and inhibition of protein synthesis was confirmed by parallel cell labelling with [^{35}S]methionine as described above. Equivalent expression levels of all constructs were confirmed by Western blots (results not shown). Only full-length HA–E1B-AP5 was methylated (Figure 2B, lane 2). No methylation signal was observed for HA–E1B-AP5ΔRGG or HA–E1B-AP5-N (Figure 2B, lanes 3 and 4), leading to the conclusion that E1B-AP5 is methylated in its RGG box *in vivo*.

Methyltransferase activity co-precipitates with E1B-AP5

To analyse whether E1B-AP5 can serve as a substrate for human arginine methyltransferases *in vitro* we used E1B-AP5 immunoprecipitated with mAb 4A11 from hypomethylated H1299 cells. Surprisingly, incubation of immunoprecipitated E1B-AP5 in *in vitro* methylation buffer including the methyl group donor SAM resulted in intense E1B-AP5 methylation without the addition of any methyltransferase activity (Figure 3A). Several faster-migrating radiolabelled bands were also observed, probably corresponding to methylated proteins that co-precipitated with E1B-AP5 (Figure 3A). No methylated proteins were observed when non-specific rat mAbs were used for immunoprecipitation (Figure 3A, lane 5). When E1B-AP5 was precipitated from H1299 cells untreated with AdOx, no labelling was detected (Figure 3A, lane 4), suggesting that most of the E1B-AP5 and co-precipitated proteins were present in these cells in fully methylated forms. The intensity of E1B-AP5 methylation and also the presence and labelling efficiency of the faster-migrating bands derived from hypomethylated cells were dependent on the stringency of the buffer used to wash the immunoprecipitated complexes with E1B-AP5 (Figure 3A), although the amount of

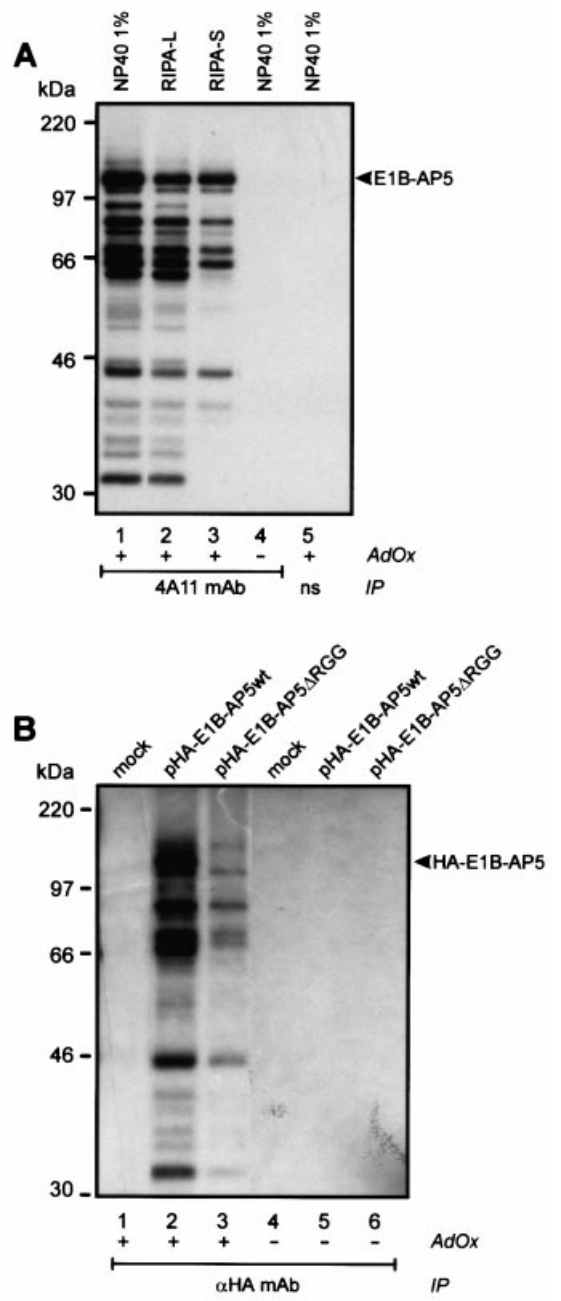

Figure 3 Methyltransferase activity is co-immunoprecipitated with E1B-AP5

(**A**) Precipitation of methyltransferase activity with endogenous E1B-AP5. H1299 cells were grown in the absence (lane 4) or presence (lanes 1, 2, 3 and 5) of AdOx. Control H1299 cells were grown without AdOx (lane 4). Immunoprecipitation was performed in buffer with increasing stringency as indicated; 300 μg of each lysate was precipitated with Protein G–4A11 mAb or Protein G–non-specific rat mAb complex. Immunoprecipitation (IP) samples were incubated with 3 μCi of SAM. Then 50% of each sample was analysed by SDS/PAGE and autoradiography; equal amounts of E1B-AP5 were confirmed in parallel by Western blotting (results not shown). Methylated E1B-AP5 is indicated by the arrow (lanes 1, 2 and 3). A 14-day exposure is shown. Abbreviation: ns, non-specific rat monoclonal antibody. (**B**) Precipitation of methyltransferase activity with transiently expressed E1B-AP5. H1299 cells were mock-transfected (lanes 1 and 4) or were transiently transfected with HA–E1B-AP5wt (lanes 2 and 5) or HA–E1B-AP5 ΔRGG (lanes 3 and 6). AdOx was added to the cell medium 1 h before transfection (lanes 1–3). Cells were lysed in RIPA-L buffer. Immunoprecipitation was performed with anti-HA mAb (αHA mAb). *In vitro* methylation was performed as above. A 14-day exposure is presented. Equal amounts of HA–E1B-AP5wt and HA–E1B-AP5 ΔRGG were confirmed by Western blotting (results not shown). The positions of molecular mass markers are indicated at the left.

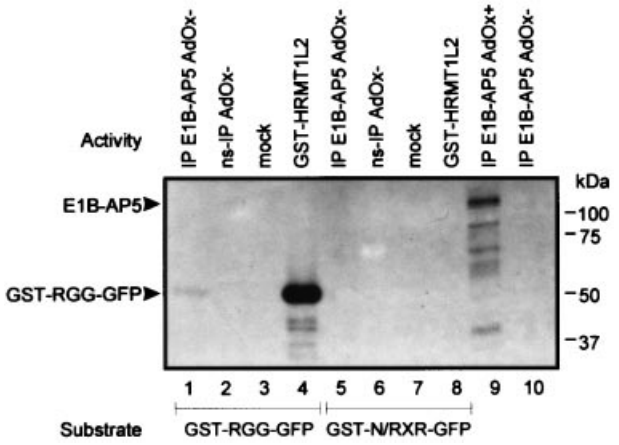

Figure 4 HRMT1L2 and E1B-AP5-associated activity methylate the recombinant RGG box of E1B-AP5

Purified GST–RGG–GFP (lanes 1–4) or GST–N/RG–GFP (lanes 5–8) (5 μg of each) were used as substrates for methylation *in vitro*. GST–HRMT1L2 (0.2 μg) was used in each reaction (lanes 4 and 8). To assay E1B-AP5-associated activity, E1B-AP5 complex was immunoprecipitated (IP) from H1299 cells with 4A11 mAb (lanes 1, 5 and 10) and non-specific rat mAb for the negative controls (lanes 2 and 6). In lanes 3 and 7 no activity was added. In lanes 9 and 10 no bacterially expressed substrate was added. E1B-AP5 precipitated out of hypomethylated H1299 cells was used as a positive control to evaluate co-precipitated methyltransferase activity (lane 9). Samples were analysed by PAGE followed by autoradiography. A 7-day exposure is shown. The positions of molecular mass markers are indicated at the right. Abbreviations: RXR, Arg-Xaa-Arg; ns, non-specific rat monoclonal antibody.

precipitated E1B-AP5 protein was equivalent in all buffer conditions (results not shown). These results suggested that the methyltransferase activity co-precipitated as a protein complex with E1B-AP5. The absence of a canonical SAM-binding site in E1B-AP5 excluded the possibility of E1B-AP5 self-methylating activity. Moreover, a recombinant baculovirus-HA-tagged E1B-AP5 immunoprecipitated from Sf9 cells did not co-precipitate any methyltransferase activity and was not methylated *in vitro* in the presence of Sf9 extracts (results not shown), suggesting that the co-precipitated methyltransferase was specific for mammalian cells.

To analyse the role of the RGG-box domain, HA-tagged E1B-AP5wt and E1B-AP5ΔRGG were transiently expressed in hypomethylated H1299 cells, immunoprecipitated with 12CA5 antibody and incubated in *in vitro* methylation buffer in the presence of SAM. Methylated E1B-AP5 was observed only with full-length protein, not with E1B-AP5ΔRGG (Figure 3B, compare lanes 2 and 3). Interestingly, co-precipitated proteins were methylated in both cases but with significantly decreased efficiency in the presence of E1B-AP5ΔRGG. This suggests that the RGG-box domain, in addition to being the site of methylation, is also involved in the formation of the E1B-AP5/methyltransferase complex immunoprecipitated from mammalian cells.

E1B-AP5 RGG-box domain is methylated *in vitro* by HRMT1L2 and the E1B-AP5-associated activity

We decided to analyse the methyl-accepting properties of the E1B-AP5 RGG box in more detail because a bacterially expressed RGG-rich fragment of human fibrillarin (GST–GAR, where GAR stands for glycine–arginine-rich) had been used successfully as a methyl-accepting substrate to assay different mammalian methyltransferase activities [15,18,29,38]. In addition, inspection

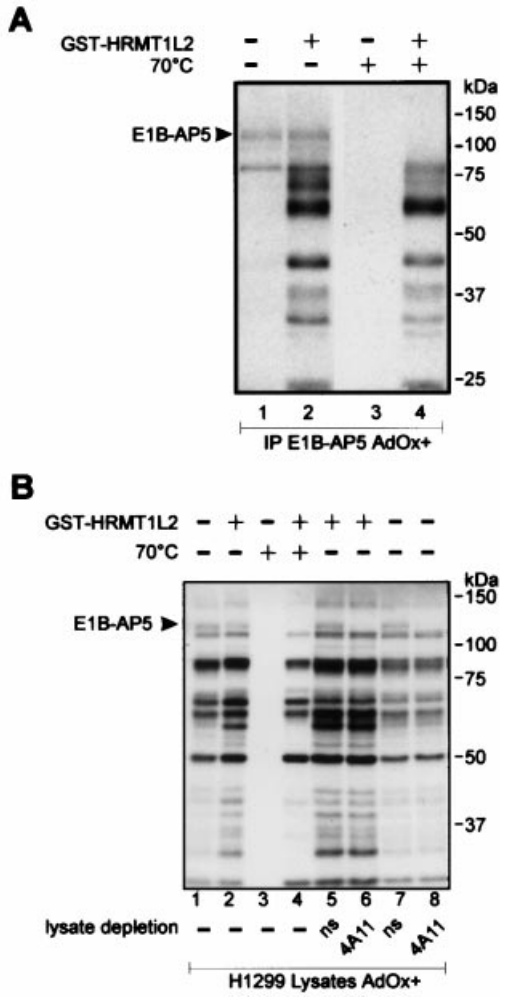

Figure 5 HRMT1L2 does not methylate endogenous E1B-AP5

(**A**) HRMT1L2 does not methylate immunoprecipitated E1B-AP5. E1B-AP5 was precipitated out of hypomethylated H1299 cells. To inactivate co-precipitated methyltransferase, half of the immunoprecipitation (IP) sample was incubated at 70 °C for 6 min (lanes 3 and 4). GST–HRMT1L2 (0.2 μg) was added to the reaction (lanes 2 and 4). Samples were analysed by PAGE followed by autoradiography. Equal amounts of E1B-AP5 in all samples were confirmed by Western blotting (results not shown). A 5-day exposure is shown. (**B**) HRMT1L2 does not contribute to E1B-AP5 methylation in cell lysates. H1299 cells were grown in the presence of AdOx for 48 h, washed twice with PBS and lysed in RIPA-L buffer; 25 μg (10 μl) of cell lysate was used in each reaction. Endogenous methyltransferase was inactivated thermally as above (lanes 3 and 4). E1B-AP5 was depleted by preincubating H1299 lysates with 4A11 mAb (lanes 6 and 8) or the control non-specific rat mAb (lanes 5 and 7). GST–HRMT1L2 (0.2 μg) was added to each reaction (lanes 2, 4, 5 and 6). A 5-day exposure is shown. Abbreviation: ns, non-specific rat monoclonal antibody. The positions of molecular mass markers are indicated at the right.

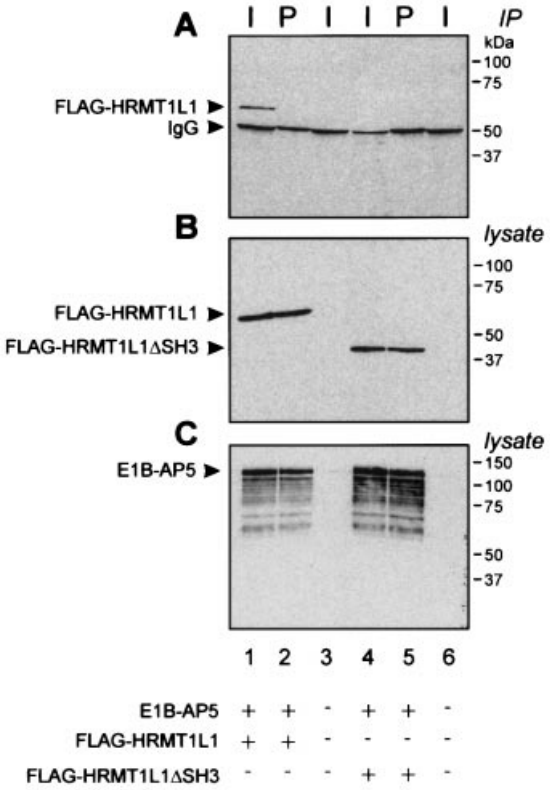

Figure 6 SH3 domain of HRMT1L1 mediates its interaction with E1B-AP5

Sf9 cells were infected with recombinant baculovirus expressing FLAG-HRMT1L1, FLAG-HRMT1L1ΔSH3 and HA–E1B-AP5 as indicated. (**A**) Immunoprecipitation (IP) was performed with rabbit anti-(E1B-AP5) serum (I) or rabbit preimmune serum (P). Immunoprecipitation samples were analysed by Western blotting with anti-FLAG mAb. Immunoprecipitation efficiency was confirmed by Western blotting with rabbit anti-E1B-AP5 serum (results not shown). FLAG-HRMT1L1wt co-precipitated with E1B-AP5 is indicated by the arrow (lane 1). (**B**) Total cell lysates were analysed for recombinant HRMT1L1 expression protein expression by Western blotting with anti-FLAG mAb. (**C**) E1B-AP5 expression was monitored by anti-(E1B-AP5) rabbit polyclonal serum. The positions of molecular mass markers are indicated at the right.

of the primary amino acid sequence of E1B-AP5 revealed that a further six potential methyl-accepting arginine residues in an Arg-Xaa-Arg context [38] are located in the N-terminal part of E1B-AP5 (Arg^{171}–Arg^{173}, Arg^{179}–Arg^{181} and Arg^{191}–Arg^{193}). Two E1B-AP5 fragments, the RGG-box domain corresponding to residues 611–688 and an N-terminal fragment (N/RG) corresponding to residues 171–193, were cloned as GST–RGG–GFP and GST–N/RG–GFP fusion proteins, expressed in *E. coli*, purified under non-denaturing conditions and used as substrates in methylation assays *in vitro*. As sources of methyltransferase activity we used either the E1B-AP5 complex immunoprecipitated with 4A11 mAb from H1299 cells or GST–HRMT1L2 purified from *E. coli*. Both were able to methylate the RGG-box domain *in vitro* (Figure 4, lanes 1 and 4) but not the N-terminal N/RG fragment (Figure 4, lanes 5 and 8). No methylation was observed when recombinant GST–GFP was used as a control substrate (results not shown). The RGG-box domain was methylated with much greater intensity by HRMT1L2 than by the E1B-AP5-associated activity. Because the amount of E1B-AP5-associated activity could not be assessed quantitatively, intensive labelling for HRMT1L2 might be explained by larger amounts of the enzyme used in the reaction.

As an alternative, we compared the efficiency of E1B-AP5-associated activity in methylating native E1B-AP5 complex from mammalian cells with that in methylating bacterially expressed GST–RGG–GFP substrate. E1B-AP5 was immunoprecipitated from H1299 cells grown in the presence or absence of AdOx. Complexes containing either 0.5 μg of E1B-AP5 purified from hypomethylated (AdOx treated) cells without additional substrate, or 0.5 μg of E1B-AP5 purified from cells not treated with AdOx mixed with 5 μg of purified GST–RGG–GFP protein, were analysed by methylation *in vitro*. Significantly, the E1B-

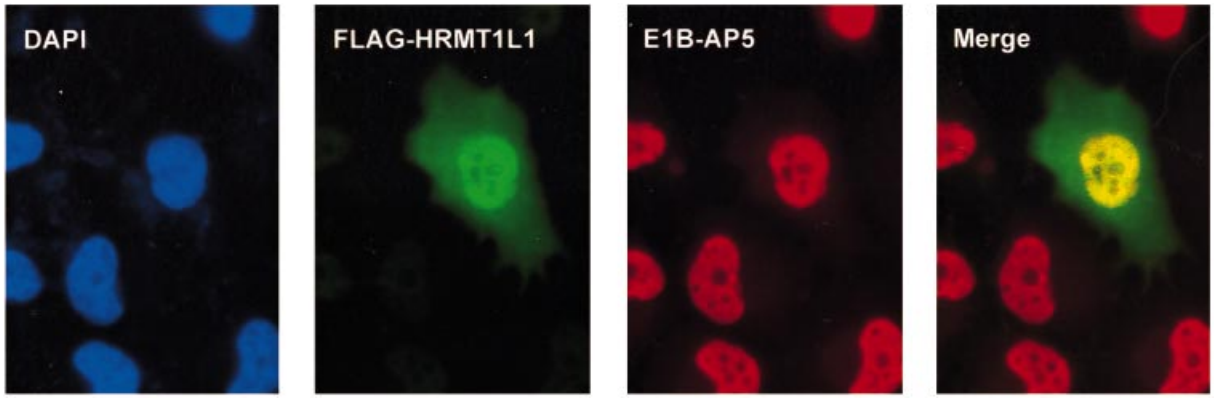

Figure 7 HRMT1L1 and E1B-AP5 co-localize in the nucleus of HeLa cells

HeLa cells were cultured on coverslips in six-well dishes, transfected with 2 μg of pFLAG-HRMT1L1 by precipitation with calcium phosphate and fixed with paraformaldehyde 24 h after transfection. Indirect immunofluorescence was performed as described in the Experimental section. Green corresponds to FLAG-tagged HRMT1L1, red indicates endogenous E1B-AP5 and yellow represents the overlay. Abbreviation: DAPI, 4,6-diamidino-2-phenylindole.

AP5-associated activity methylated full-length E1B-AP5 much more efficiently than the bacterially derived GST–RGG–GFP substrate (Figure 4, compare lanes 1 and 9). Moreover, the other proteins co-precipitating with E1B-AP5 (each protein less than 0.1 μg) were also more efficiently methylated than the added recombinant substrate. We conclude that the enzymic activity of E1B-AP5-associated methyltransferase might depend on substrate–protein complex formation and/or might be more sensitive than other methyltransferases to the protein conformational context of the RGG box.

HRMT1L2 is unable to methylate endogenous E1B-AP5

Because HRMT1L2 methylated an artificial substrate containing the RGG-box domain of E1B-AP5 extremely efficiently, the next question was whether HRMT1L2 could methylate endogenous E1B-AP5. E1B-AP5 was precipitated from H1299 cells grown in the presence of AdOx with mAb 4A11. Methylation of immunoprecipitated E1B-AP5 was not altered by the addition of HRMT1L2 in a methylation reaction *in vitro* (Figure 5A, lanes 1 and 2), indicating that HRMT1L2 was unable to hypermethylate native E1B-AP5 further than the intrinsic E1B-AP5-associated methyltransferase. However, the methylation intensity of several proteins co-precipitating with E1B-AP5 was markedly increased when HRMT1L2 was added (Figure 5A, lane 2). It was important then to test whether HRMT1L2 activity was masked by, and subsequently could substitute for, the E1B-AP5-associated methyltransferase. Enzyme activity that was co-precipitated with E1B-AP5 was inactivated by heating samples to 70 °C for 6 min before adding HRMT1L2 to the methylation reaction *in vitro*. E1B-AP5 was not methylated under these conditions (Figure 5A, lanes 3 and 4), in contrast with the intense methylation of other co-precipitated proteins.

We also analysed the ability of HRMT1L2 to methylate E1B-AP5 in H1299 cell lysates. The addition of HRMT1L2 to cell lysates did not increase the intensity of the 120 kDa band (Figure 5B, compare lanes 1 and 2, 5 and 7). HRMT1L2 also did not methylate the corresponding 120 kDa protein in heat-inactivated cell lysates (Figure 5B, lanes 3 and 4). The identity of the 120 kDa band was confirmed as E1B-AP5 by immunodepletion involving the preincubation of lysates with mAb 4A11 (Figure 5B; note the absence of the 120 kDa band in lanes 6 and 8).

Identification of HRMT1L1 as an E1B-AP5 interaction protein in two-hybrid screen

On the basis of these results we conclude that E1B-AP5-associated methyltransferase (1) is not HRMT1L2 and (2) apparently needs to form a complex with the substrate to exhibit activity, in other words it can interact directly with E1B-AP5. Yeast two-hybrid screening was performed to identify the potential E1B-AP5-interacting methyltransferase. A HeLa cDNA library was screened with pASE1B-AP5-C expressing the GAL4-DNA-binding domain fused to a central fragment of E1B-AP5 comprising residues 213–732. After double selection with histidine/β-galactosidase, 16 independent clones were sequenced. Database analysis revealed that one of these clones contained cDNA with 100% similarity to the human methyltransferase HRMT1L1 cDNA region encoding residues 1–97 [27]. HRMT1L1 is the only arginine methyltransferase family member containing an SH3 domain, located between residues 31 and 84, and therefore present in the methyltransferase fragment interacting with E1B-AP5 in the yeast two-hybrid system.

HRMT1L1 interacts with E1B-AP5 through its SH3 domain

To analyse interactions of E1B-AP5 and HRMT1L1 *in vivo* we generated recombinant baculoviruses expressing FLAG-tagged full-length HRMT1L1 (HRMT1L1wt) or HRMT1L1 lacking the SH3 domain (HRMT1L1ΔSH3). After infection of Sf9 cells with a combination of baculoviruses expressing HA-tagged E1B-AP5 and HRMT1L1, protein–protein interactions were analysed by co-immunoprecipitation. HRMT1L1wt and HRMT1L1ΔSH3 were expressed equally well (Figure 6B, lanes 1, 2, 4 and 5) but only HRMT1L1wt was co-precipitated with rabbit polyclonal anti-(E1B-AP5) serum, indicating that the protein interaction was mediated by the SH3 domain (Figure 6A, lane 1). Identical results were observed with anti-HA 12CA5 antibody for immunoprecipitation (results not shown). In the converse experiment, with anti-FLAG antibody for immunoprecipitation, E1B-AP5 co-precipitated with HRMT1L1wt but not with HRMT1L1ΔSH3 (results not shown).

HRMT1L1 co-localizes with E1B-AP5 in the nucleus

To examine further the interaction of E1B-AP5 and HRMT1L1 *in vivo* we analysed their co-localization in mammalian cells.

HeLa cells growing on coverslips in six-well dishes were transiently transfected with FLAG-tagged HRMT1L1 and subjected to an indirect immunofluorescence assay. Fixed cells were incubated with a mixture of rat monoclonal anti-(E1B-AP5) and mouse monoclonal anti-FLAG antibodies and then stained by a combination of FITC-conjugated anti-mouse and Texas Red anti-rat antibodies. Endogenous E1B-AP5 is stained red and is localized exclusively in the nucleus. HRMT1L1 is stained green and is localized in the nucleus and cytoplasm, but nuclear staining is predominant. Overlaying the same red and green stained cell results in yellow staining where protein co-localization occurs. Most of the nuclear fraction of HRMT1L1 was co-localized with E1B-AP5 (Figure 7). We also observed that deletion of the SH3 domain significantly decreased the expression levels of HRMT1L1 and resulted in weak staining that was equally distributed throughout the nucleus and cytoplasm (results not shown). We conclude that E1B-AP5 and HRMT1L1 can form a complex requiring the HRMT1L1 SH3 domain *in vivo* in human cells.

DISCUSSION

In the present study we examined the arginine methylation of E1B-AP5, a protein that is proposed to be involved in nucleocytoplasmic mRNA transport [4] and, because of its similarity to hnRNP-U and hnRNP-G, considered to be a new member of the hnRNP family. To distinguish between E1B-AP5 and its closest analogue, hnRNP U, particularly because both have similar electrophoretic migration properties, an anti-(E1B-AP5) mAb was generated that did not cross-react with hnRNP U. Methylation of E1B-AP5 *in vivo* was demonstrated by immunoprecipitation of the protein with an mAb out of metabolically methylated H1299 cells followed by gel resolution. Additionally, results were confirmed by similar experiments with H1299 cells stably expressing an HA-tagged version of E1B-AP5.

A characteristic feature of most of hnRNPs and several other proteins involved in RNA metabolism is the presence of GAR regions (RGG-box domains) and several lines of evidence indicate that RGG boxes are responsible for hnRNP methylation. Asymmetric methylation *in vivo* of arginine residues located in a GAR region of hnRNP A1 was demonstrated directly [39,40]. The bacterially purified GST-fused GAR domain of fibrillarin serves as a perfect substrate for several mammalian type 1 arginine methyltransferases *in vitro* [15,18,29,38]. Synthetic peptide corresponding to the RGG box of hnRNP-U is methylated *in vitro* by partly purified human methyltransferase [36]. An additional interesting example is the herpes simplex virus RNA-binding protein ICP27. Analysis of the wild-type virus and a mutant virus with a deletion covering the RGG-box domain of ICP27 revealed that methylation of ICP27 occurs only in the presence of the RGG-box domain [41].

E1B-AP5 contains an extensive RGG-box domain located in the C-terminal part of the protein, although the sequence does not correspond exactly to the proposed consensus [15,39]. To analyse the role of the RGG domain in the methylation of E1B-AP5 *in vivo*, we designed an assay combining transient expression of HA-tagged E1B-AP5 constructs with methylation/immunoprecipitation *in vivo*. Deletion of the RGG-box domain abolished E1B-AP5 methylation *in vivo*, indicating that the C-terminal RGG-box domain is responsible for E1B-AP5 methylation *in vivo*.

Studying the poly(A)-binding protein II from calf thymus revealed new unusual sites for arginine methylation comprising clustered Arg-Xaa-Arg motives [38]. The N-terminal part of E1B-AP5 also contains three closely located Arg-Xaa-Arg motives (Arg^{171}–Arg^{173}, Arg^{179}–Arg^{181} and Arg^{191}–Arg^{193}). Therefore we also analysed the methylation of E1B-AP5 *in vitro* with a bacterially expressed recombinant RGG-box domain and the N-terminal Arg-Xaa-Arg region as substrates. Only the RGG-box domain was methylated by HRMT1L2 or the E1B-AP5 co-precipitated activity, suggesting that the RGG-box domain of E1B-AP5 is solely responsible for its methylation.

The biological role of arginine methylation in hnRNP is still unclear, although several well-argued functions have been proposed, usually on the basis of indirect evidence. The involvement of arginine methylation in protein–RNA interaction is supported by the fact that the RGG-box domains, besides being sites for arginine methylation, commonly contribute to protein–RNA interaction (reviewed in [15]). Additionally, the methylated form of hnRNP A1 was observed to exhibit weaker binding to single-stranded DNA-cellulose than the non-methylated form [25]. We also found that the RGG-box domain mediated E1B-AP5 binding to RNA *in vitro* (results not shown), suggesting that arginine methylation can modulate this interaction.

Another possibility is that the interaction of E1B-AP5 with some protein partner is regulated by arginine methylation. E1B-AP5 contains a polyproline stretch immediately after the RGG-box domain. Proline-rich regions have been widely demonstrated to be involved in protein–protein interactions (reviewed in [42]). In particular, SH3 and WW protein-interaction domains prefer ligand sequences that are proline-rich. As shown recently [19], protein arginine methylation can selectively modulate the binding of proline regions to SH3 and WW domains. Further identification of proteins binding to E1B-AP5 through its proline-rich region would permit an analysis of the significance of arginine methylation in protein–protein interaction.

To analyse E1B-AP5 methylation in closer detail we used an assay *in vitro* with immunoprecipitates from hypomethylated H1299 cells. Interestingly, methyltransferase activity was found to form a complex with E1B-AP5 *in vivo* and the activity seemed to be specific for mammalian cells.

Human HRMT1L2 is a true homologue of the well-analysed rat PRMT1, which was shown to be a predominant mammalian methyltransferase [43]; it probably corresponds to the partly purified arginine methyltransferase described by Liu and Dreyfuss [36]. We first considered the possibility that this enzyme is responsible for E1B-AP5-specific methylation. However, despite intensive methylation of the recombinant bacterially expressed E1B-AP5 RGG-box domain, HRMT1L2 failed to methylate full-length E1B-AP5 in cell lysates or immunoprecipitated endogenous E1B-AP5.

Because several mammalian arginine methyltransferases were identified by interaction with different cellular regulatory proteins in the yeast two-hybrid system [16,18,26,28], we performed two-hybrid screening to define potential interaction of arginine methyltransferase with E1B-AP5. One of the proteins found was HRMT1L1. HRMT1L1, which is considered to be a methyltransferase because it has sequence similarities with yeast Hmt1 and other arginine methyltransferases [27], contains an SH3 domain, known to mediate protein–protein interaction [32–34]. The fact that the fragment encoded by the cDNA clone isolated in the two-hybrid screen contained the SH3 domain suggested that this domain mediates the interaction of HRMT1L1 with E1B-AP5. Analysis of this protein–protein interaction in a baculovirus expression system revealed that the SH3 domain of HRMT1L1 is indeed necessary for interaction with E1B-AP5 *in vivo*. Moreover, interaction of E1B-AP5 and HRMT1L1 was confirmed by immunofluorescence studies. Most of the HRMT1L1 in the nuclear fraction co-localized with E1B-AP5,

indicating that the complex can exist *in vivo* in human cells. Deletion of the SH3 domain resulted in a significant decrease in HRMT1L1 expression levels and nuclear localization (results not shown), suggesting that the SH3 domain, in addition to mediating protein–protein interactions, contributes to the stability of HRMT1L1. To analyse the E1B-AP5/HRMT1L1 complex further, we attempted to design several systems *in vitro*. However, neither *E. coli*- nor baculovirus-expressed HRMT1L1 exhibited methyltransferase activity in combination with E1B-AP5. We also observed that in a baculovirus protein–protein interaction assay, increasing the buffer stringency led to a marked decrease in complex formation, although the most stringent conditions used for E1B-AP5 immunoprecipitation from mammalian cells did not significantly affect the amount of co-precipitated methyltransferase activity. Because SH3 domains usually mediate highly specific but transient low-affinity protein–protein interactions [32], we surmise that binding of HRMT1L1 to E1B-AP5 *in vivo* might be stabilized by other mammalian-specific complex components or protein modifications. Moreover, we presume that HRMT1L1 can exhibit its enzymic activity only as a component of a multiprotein aggregate. Reconstitution of this complex would be needed to demonstrate the HRMT1L1 activity *in vitro*. Our results strongly suggest that E1B-AP5 exists *in vivo* in a complex with HRMT1L1, which is the methyltransferase responsible for its specific methylation.

PRMTs have been identified as interacting with diverse range of cellular regulatory proteins [16,20,26,28,30]. Being components of ligand-induced signal transduction, PRMTs act at the level of transcriptional activation. Several mechanisms of PRMT-dependent co-activation of transcription were demonstrated recently [16,20,23,24]. We can consider HRMT1L1 as a potential transcriptional co-activator. We presume that E1B-AP5 not only serves as a specific substrate for HRMT1L1 but can also regulate the enzyme's activities, as shown for the PRMT1–(interleukin enhancer-binding factor 3) complex [18]. The most similar to E1B-AP5, hnRNP U, has been shown to bind a nuclear hormone receptor (glucocorticoid receptor) and to inhibit the dexamethasone-induced transcriptional activation of glucocorticoid receptor [44]. The extensive similarity between E1B-AP5 and hnRNP U suggests closely related functions. It would therefore be of great interest (1) to investigate possible transcriptional activities of E1B-AP5 and to determine whether it can support or antagonize the transcriptional repression of hnRNP U; (2) to analyse whether hnRNP U can interact with HRMT1L1; and (3) to test the hypothesis that E1B-AP5 is a potential transcriptional regulator that can specifically modulate HRMT1L1 activity.

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