AUF1/hnRNP D is a novel protein partner of the EBER1 noncoding RNA of Epstein-Barr virus

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ABSTRACT

Epstein-Barr virus (EBV)-infected cells express two noncoding RNAs called EBV-encoded RNA (EBER) 1 and EBER2. Despite their high abundance in the nucleus (about 10^6 copies), the molecular function of these noncoding RNAs has remained elusive. Here, we report that the insertion into EBER1 of an RNA aptamer that binds the bacteriophage MS2 coat protein allows the isolation of EBER1 and associated protein partners. By combining MS2-mediated selection with stable isotope labeling of amino acids in cell culture (SILAC) and analysis by mass spectrometry, we identified AUF1 (AU-rich element binding factor 1)/hnRNP D (heterogeneous nuclear ribonucleoprotein D) as an interacting protein of EBER1. AUF1 exists as four isoforms generated by alternative splicing and is best known for its role in destabilizing mRNAs upon binding to AU-rich elements (AREs) in their 3' untranslated region (UTR). Using UV crosslinking, we demonstrate that predominantly the p40 isoform of AUF1 interacts with EBER1 *in vivo*. Electrophoretic mobility shift assays show that EBER1 can compete for the binding of the AUF1 p40 isoform to ARE-containing RNA. Given the high abundance of EBER1 in EBV-positive cells, EBER1 may disturb the normal homeostasis between AUF1 and ARE-containing mRNAs or compete with other AUF1-interacting targets in cells latently infected by EBV.

Keywords: AUF1; EBER1; noncoding RNA

INTRODUCTION

Epstein-Barr virus (EBV) is a human γ -herpesvirus that is associated with B-cell malignancies, such as Burkitt's and Hodgkin's lymphomas, as well as with several epithelial cancers, including nasopharyngeal and gastric carcinomas (Young and Rickinson 2004). Encoded in the EBV genome are two evolutionary conserved noncoding RNAs called EBV-encoded RNA (EBER) 1 and EBER2, which are non-polyadenylated, RNA polymerase III transcripts of 167 and 172 nucleotides (nt), respectively. EBERs accumulate to over 10^6 copies in the nucleus during all stages of latent infection (Lerner et al. 1981; Rosa et al. 1981; Glickman et al. 1988).

Although the molecular mode of action of EBERs is not understood, phenotypic observations point to a role in EBV oncogenicity. The expression of EBERs in B lymphocytes results in colony formation in soft agar and tumor induction in nude mice (Komano et al. 1999; Ruf et al. 2000). Recent reports have shown that EBER1 alone is sufficient to elicit these phenotypes (Houmani et al. 2009; Repellino et al.

2010). Moreover, EBERs have been suggested to enhance growth transformation efficiency in EBV-infected B lymphocytes (Yajima et al. 2005). This point remains controversial, however, as other studies have concluded that EBERs do not affect EBV-induced growth transformation (Swaminathan et al. 1991; Gregorovic et al. 2011). Transcription profile changes that contribute to tumor formation after expression of EBERs have also been reported, such as up-regulation of various cytokines depending on the cell type: interleukin-10 in B lymphocytes, interleukin-9 in T cells, and insulin-like growth factor 1 in nasopharyngeal carcinoma cells (Kitagawa et al. 2000; Iwakiri et al. 2003; Yang et al. 2004).

Originally, EBERs were discovered to form stable ribonucleoprotein (RNP) complexes with the cellular protein La (Lerner et al. 1981), which binds all nascent RNA polymerase III transcripts and aids in their maturation (Wolin and Cedervall 2002). The large ribosomal subunit protein L22 was subsequently identified as another partner of EBER1 (Toczyski and Steitz 1991; Toczyski et al. 1994). No specific ligands for EBER2 have yet been identified.

Here, we aimed to provide further insight into the molecular mode of action of the EBER1 RNP by identifying novel RNA-protein interactions. We selected EBER1 RNPs from cell extracts using complementary oligonucleotides and

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RNA aptamers, which are nucleotide sequences with strong affinity for specific molecules (Srisawat and Engelke 2002; Zhou et al. 2002). We show that insertion of a widely used RNA aptamer for the bacteriophage MS2 coat protein enables the isolation of EBER1 and associated proteins. Combining this aptamer-mediated selection with mass spectrometry analysis, we identified AU-rich element binding factor 1 (AUF1)/heterogeneous nuclear RNP D (hnRNP D) as a novel interacting protein of EBER1 both in vitro and in vivo. AUF1 is best known for its ability to bind AU-rich elements (AREs), which commonly reside in 3' untranslated regions (UTRs), and to enhance the decay of many short-lived mRNAs, including those of proto-oncogenes, cell cycle regulators, and cytokines (Caput et al. 1986; Chen and Shyu 1995; Loflin et al. 1999). However, stabilizing effects of AUF1 have also been reported (Xu et al. 2001), as well as diverse functions in aging, telomere maintenance, and suppression of senescence (Pont et al. 2012). AUF1 is expressed as four isoforms (p37, p40, p42, p45) that originate from alternative splicing events and have been ascribed nonredundant functions (Wagner et al. 1998; Arao et al. 2000; Sarkar et al. 2003; Raineri et al. 2004; Kedar et al. 2012). Given the observation that AUF1 overexpression leads to tumorigenesis (Gouble et al. 2002), the interplay between AUF1 and EBER1 could contribute to the oncogenic phenotype of EBER1-expressing cells.

RESULTS

Selection of EBER1 RNP complexes using an RNA aptamer for the bacteriophage MS2 coat protein

To identify novel EBER1-interacting proteins, we sought a system that facilitates the direct capture of EBER1 from cell lysates. We used BJAB cells, an EBV-negative Burkitt's lymphoma B-cell line, and BJAB-B1 cells, which are derived from BJAB cells by in vitro infection with EBV (Menezes et al. 1975; Fresen et al. 1977). First, we investigated complementary oligonucleotides as a tool to select EBER1. To identify accessible region(s) of EBER1, RNase H cleavage assays were performed by adding seven ~30-nt-long antisense DNA oligonucleotides to BJAB-B1 cell lysate to induce EBER1 digestion by endogenous RNase H (Supplemental Fig. S1; Wassarman and Steitz 1991). Only the oligonucleotide complementary to stem-loop III of EBER1 induced RNase H digestion, indicating that this region is partially available in the RNP. A 2'-O-methyl modified RNA oligonucleotide complementary to stem-loop III was covalently attached to adipic acid dihydrazide Agarose beads (Borah et al. 2009) and incubated with EBV-positive BJAB-B1 cell lysate (Fig. 1A). Although we were able to select ~5% of total EBER1 RNA, known interacting proteins of EBER1, such as La and L22, were not coselected (Fig. 1D).

Next, we attempted to select EBER1 by inserting a single S1-aptamer, which has high affinity for streptavidin (Srisawat

and Engelke 2002), into several sites in the RNA. Insertion only into stem-loop IV preserved the affinity of S1 for streptavidin, whereas insertions into the 5'- or 3'-end or into stem-loop III did not (Fig. 1B; data not shown). BJAB cells that express EBER1 containing the S1-aptamer (EBER1-S1) were generated by lentiviral transduction (Cao et al. 2008). Approximately 5% of total EBER1-S1 RNA could be captured on streptavidin beads from lysate prepared from these cells. However again, the EBER1-interacting proteins La and L22 failed to be coselected (Fig. 1D).

Finally, we inserted three MS2-RNA hairpins, which bind the MS2 bacteriophage coat protein (Zhou et al. 2002), at the 5'- or 3'-end as well as into stem-loop IV of EBER1, and generated BJAB cells expressing these constructs by lentiviral transduction (Cao et al. 2008). The BJAB cells expressing EBER1 with MS2-hairpin insertions in stem-loop IV (EBER1-MS2) (Fig. 1C) exhibited a 12-fold higher expression level than the other EBER1-MS2 constructs (data not shown). The addition of MS2 coat protein recombinantly expressed in bacteria as a fusion protein with maltose binding protein (MBP) to the lysate with amylose resin recovered up to 20% of EBER1-MS2 RNA. More importantly, EBER1-interacting proteins La and L22 were coselected (Fig. 1D).

SILAC coupled with mass spectrometry identifies novel protein partners of EBER1

To investigate proteins purified by EBER1-MS2 selection, we resolved the maltose eluate by SDS-gel electrophoresis and compared the eluted proteins to those from lysate containing unmodified EBER1. Although the silver-stained protein profiles revealed no obvious differences, the known EBER1 interactors La and L22 were specifically enriched only in the EBER1-MS2 containing lysate upon Western blot analysis (Supplemental Fig. S2).

To distinguish interacting proteins from the background of nonspecific contaminants, SILAC (stable isotope labeling of amino acids in cell culture) in combination with mass spectrometry analysis was employed (Blagoev et al. 2003; Mann 2006). In this method, cell lines are cultured in medium containing the essential amino acids arginine and lysine bearing either naturally abundant ^{12}C , ^{14}N (light) or ^{13}C , ^{15}N (heavy) isotopes. After selection, specific binding proteins are identified by mass spectrometry through the relative enrichment of peptides containing the isotope originating from one cell line, whereas contaminating proteins yield light and heavy peptides in equal abundance. EBER1 and EBER1-MS2 expressing cells were cultured in light and heavy medium, respectively, and equal amounts of lysate were mixed prior to MS2-aptamer selection. EBER1-MS2 RNP complexes were eluted from amylose resin by the addition of maltose, and the proteins were size-fractionated by SDS-gel electrophoresis prior to mass spectrometry (Fig. 2A). MaxQuant software was used to determine the SILAC

FIGURE 1. The MS2-aptamer selects EBER1-interacting proteins. (A–C) Three strategies for selecting EBER1 RNPs are depicted. (A) EBER1 was isolated using a complementary oligonucleotide covalently linked to adipic acid dihydrazide Agarose beads. Alternatively, an aptamer was inserted into stem-loop IV of EBER1. (B) The S1-aptamer (45 nt) or (C) three MS2-hairpin loops (82 nt) were used to select EBER1 RNPs on streptavidin beads or on MS2 coat-MBP fusion protein tethered to amylose resin, respectively. (D) For selection with complementary oligonucleotides, BJAB-B1 cell lysate was used. For aptamer-mediated selections, stable BJAB cell lines were generated that express either EBER1-S1 or EBER1-MS2. A Northern blot probed for EBER1 assessed the pull-down efficiency of aptamer-containing EBER1 RNAs versus unmodified EBER1. Western blots using antibodies against La and L22 asked whether known EBER1-interacting proteins were coselected.

ratio of eluted proteins, thus identifying candidate interacting proteins (Supplemental Table S1). Among the proteins that displayed SILAC ratios characteristic of specific interactors were the previously established EBER1-interacting proteins La and L22, as well as AUF1 (Supplemental Fig. S3).

We confirmed AUF1 as an EBER1-interacting protein using anti-AUF1 antibodies (Pinol-Roma et al. 1988). There are four AUF1 spliced isoforms: All contain two RNA recognition motifs and a C-terminal glutamine-rich domain, but differ by the presence or absence of two regions called inserts A and B (Fig. 2B). MS2-aptamer selection predominantly captured the p40 and/or p42 isoform of AUF1, which comigrates in the gel, and, more weakly, the p45 isoform (Fig. 2C). The p37 isoform was not selected. Lysate containing unmodified EBER1 provided a negative control.

EBER1 predominantly interacts with the p40 isoform of AUF1 in vivo

To demonstrate that the interaction of AUF1 with EBER1 occurs in vivo, we used a previously developed method that entails UV crosslinking of intact cells, immunoprecipitation, and washing under stringent conditions (Cook et al.

2004). The AUF1 peptide sequence identified by mass spectrometry analysis could not discriminate between the four isoforms, as it lies within the RNA recognition motif II common to all isoforms (Fig. 2B). We therefore individually cotransfected into HEK293T cells plasmids expressing a Flag-tagged version of each AUF1 isoform as well as an EBER1 plasmid, UV-irradiated and coimmunoprecipitated crosslinked EBER1 using anti-Flag antibodies (Fig. 3A). While all isoforms of AUF1 detectably bound EBER1, compared to coprecipitation in the absence of UV crosslinking, the p40 isoform showed the most pronounced interaction (Fig. 3B). To distinguish between preferential binding of the p40 AUF1 isoform to EBER1 and enhanced crosslinking efficiency of this isoform, we repeated the immunoprecipitation of Flag-tagged p40^{AUF1} in the presence of Myc-tagged p40 and p45 isoforms. If the p40 isoform is preferentially bound to EBER1, only the Myc-tagged p40 isoform should interfere with EBER1 coimmunoprecipitation by anti-Flag antibodies. Indeed, Figure 3C shows a decrease in EBER1 coimmunoprecipitation in the presence of Myc-tagged p40^{AUF1} compared with coexpression of Myc-tagged p45^{AUF1}, arguing that the p40 isoform preferentially binds EBER1.

An in vivo interaction of the p40 isoform and EBER1 is supported by the fact that both molecules are found in the

FIGURE 2. AUF1/hnRNP D is a novel EBER1-interacting protein. (A) Experimental outline of the EBER1-MS2 selection coupled to SILAC and mass spectrometry analysis. (B) Schematics of the four isoforms of AUF1 (Wagner et al. 1998), which originate from alternative splicing and differ by the presence or absence of two regions called Insert A and Insert B. The peptide sequenced by tandem mass spectrometry analysis is part of the RNA recognition motif (RRM) II domain as indicated at the bottom. Q-rich indicates glutamine-rich domain. (C) Confirmation of the specificity of the EBER1-MS2 selection of AUF1. Lysates of BJAB cells containing either EBER1 or EBER1-MS2 were subjected to MS2-mediated selection. RNA was isolated and probed for EBER1 by Northern blotting to determine the pull-down efficiency. Western blots for AUF1 showed that AUF1 is selected only from cell lysates containing EBER1-MS2 and not from lysates containing unmodified EBER1. The four isoforms of AUF1 are indicated; the p40 and p42 isoforms comigrate in the gel. The asterisk indicates a cross-reactive band. Western blot analysis of L22 is shown as a positive control.

same subcellular compartment (the p40 isoform localizes to both cytoplasm and nucleus [Xu et al. 2001], whereas EBER1 is strictly nuclear [Fok et al. 2006a]). To confirm the *in vivo* interaction of endogenous AUF1 and EBER1 in EBV-positive B lymphocytes, we used Raji cells, which are Burkitt's lymphoma cells naturally infected with EBV (Drexler and Minowada 1998), and antibodies against AUF1 (Dempsey et al. 1998) as well as L22, as a positive control for *in vivo* UV crosslinking and subsequent immunoprecipitation. EBER1 was coimmunoprecipitated only upon UV irradiation with anti-AUF1 and, as expected, anti-L22 antibodies (Fig. 3D, upper panel). We probed the same Northern blot for EBER2 as a specificity control, and observed no binding for either protein (Fig. 3D, lower panel). We conclude that EBER1 interacts *in vivo* with the p40 isoform of AUF1.

EBER1 can compete with the binding of AUF1 to ARE RNA *in vitro*

Since AUF1 binds the 3' UTRs of ARE-containing mRNAs to regulate their stability (Chen and Shyu 1995; Loflin et al. 1999), we asked whether AUF1 interaction might affect the level of EBER1 in EBV-infected cells. We generated BJAB-B1 cells that express a doxycycline-inducible short hairpin RNA that reduces the amounts of all isoforms of AUF1 protein to ~10% of the endogenous level (Supplemental Fig. S4A). Despite this marked depletion of AUF1, EBER1 levels in EBV-infected cells remained unaltered 5 d post

doxycycline treatment, suggesting that AUF1 does not affect the stability of EBER1 (Supplemental Fig. S4B).

We also examined the possibility that EBER1 interferes with the interaction between AUF1 and ARE-containing mRNAs. We *in vitro* transcribed 38 nt of the core ARE from the TNF α -mRNA as well as EBER1 and performed electrophoretic mobility shift assays with recombinant p40^{AUF1} protein (see Materials and Methods). The TNF α -ARE formed a single RNP complex with p40^{AUF1}, as previously described (Fig. 4A; Zucconi et al. 2010). EBER1 formed four distinct RNP complexes with p40^{AUF1}, suggesting that multiple regions of EBER1 interact with AUF1 (Fig. 4B). This behavior is reminiscent of RNP complex formation between EBER1 and L22, in which multiple stem-loops within EBER1 contribute to the RNA-protein interaction (Fok et al. 2006b). AUF1 and L22, however, form distinctly different RNP complexes with EBER1, since single stem-loop deletions did not result in a decrease in the overall number of AUF1-EBER1 RNP complexes (data not shown), in contrast to L22 (Fok et al. 2006b). EBER1 and the TNF α -ARE formed RNP complexes at comparable AUF1 concentrations (Fig. 4, cf. A and B). Accordingly, we observed that a 2.5-fold molar excess of EBER1 effectively competes with the ARE for binding to p40^{AUF1} in bandshift assays (Fig. 4C). To corroborate the specificity of EBER1 in this competition, we used the adenoviral VAI RNA, another viral noncoding RNA of similar length (~160 nt) transcribed by RNA polymerase III (Steitz et al. 2011), and

FIGURE 3. EBER1 binds predominantly the p40 isoform of AUF1 in vivo. (A) Experimental outline for UV crosslinking and coimmunoprecipitation of EBER1 to detect in vivo interactions. (B) The four Flag-tagged isoforms of AUF1 were transiently expressed individually, along with EBER1, in HEK293T cells. Immunoprecipitation using anti-Flag antibodies was carried out either omitting or including UV crosslinking, followed by RNA isolation and detection of EBER1 by Northern blot. All AUF1 isoforms bind EBER1 in vivo, with the p40 isoform showing the strongest crosslinking efficiency. Anti-Flag antibodies were used in a Western blot to verify the expression of each AUF1 isoform. (C) Competition experiments were carried out, showing that the enhanced interaction of p40^{AUF1} with EBER1 is due to preferential binding rather than preferential crosslinking efficiency. Flag-p40^{AUF1} and EBER1 were coexpressed in HEK293T cells with either an empty vector, Myc-tagged p40^{AUF1}, or Myc-tagged p45^{AUF1}, and immunoprecipitation was carried out using anti-Flag antibodies. The plasmids expressing Myc-tagged AUF1 were transfected in 10-fold molar excess over the Flag-tagged isoform. Western blots using anti-Flag or anti-Myc antibodies were carried out to show comparable expression of Flag-p40^{AUF1} or the Myc-tagged AUF1 isoforms. (D) In vivo UV crosslinking of Raji cells was followed by immunoprecipitation using anti-L22 (as a positive control) and anti-AUF1 antibodies, and EBER1 was detected by Northern blot analysis. The same blot was probed for EBER2 as a specificity control.

observed a decrease in ARE binding only at a much higher molar excess (25-fold) (Fig. 4D). Thus, high levels of EBER1 could potentially interfere with AUF1 binding to cellular ARE-containing mRNAs in EBV-transformed cells.

DISCUSSION

Although several EBER1-interacting proteins have been identified to date, the molecular mode of action and, in particular, the oncogenic influence of the EBER1 RNP remain poorly understood. In an attempt to identify novel interacting partners that might shed light on the molecular function of the EBER1 RNP, we established a method employing the MS2-hairpin aptamer to capture EBER1 RNPs

directly from cell lysates. By combining EBER1-MS2 selection with mass spectrometry, AUF1 was identified as a novel interacting partner of EBER1. Preferential binding of the p40 isoform relative to other AUF1 isoforms in vivo provided evidence of a specific interaction. Our observation that EBER1 can compete for the binding of AUF1 to a classical ARE RNA in electrophoretic mobility shift assays suggests a physiologically relevant function for this interaction.

Many other RNPs have been captured utilizing complementary oligonucleotides (Wassarman and Steitz 1991; Borah et al. 2011; Simon et al. 2011), an approach whose primary advantage is that no modifications to the RNA are required. Surprisingly, although EBER1 could be selected by an antisense oligonucleotide, La and L22, the best-characterized EBER1 interacting partners, were not coselected. The region of EBER1 we identified as a target for hybridization and RNase H cleavage overlaps with stem-loop III, previously characterized as the major binding site for L22 (Toczyski and Steitz 1993; Fok et al. 2006b). Whereas in prior immunoprecipitation experiments >95% of total EBER1 was precipitated by anti-L22 antibodies (Toczyski and Steitz 1993), oligonucleotide selection captured only 5% (Fig. 1D), suggesting that only EBER1 molecules not associated with L22 are isolated by this method. Similar to selection with complementary oligonucleotides, S1-aptamer-tagged EBER1 was retained on streptavidin beads, but again neither La nor L22 was copurified. Inserting RNA aptamers has the inherent risk of rendering RNAs

nonfunctional, particularly in case of noncoding RNAs, where both sequence and structure can be critical for function. It is possible that the addition of nucleotides disrupts the proper assembly of EBER1 with La and L22. However, we were able to coimmunoprecipitate EBER1-S1 using anti-L22 antibodies (data not shown), supporting the existence of two distinct pools of EBER1-S1: one that binds L22 but cannot be isolated by streptavidin, and one that retains affinity for streptavidin but does not bind L22.

Several interacting proteins were identified by EBER1-MS2 selection coupled to mass spectrometry. In addition to AUF1, two other hnRNP proteins (hnRNP A1 and hnRNP A2/B1) exhibited SILAC ratios indicating association with EBER1 (Supplemental Table S1). In the case of AUF1, we

FIGURE 4. EBER1 can compete with the binding of AUF1 to ARE RNA in vitro. (A,B) End-labeled TNF α -ARE RNA (A) or EBER1 (B) was incubated at 2 nM with increasing amounts (from 50 to 1000 nM) of recombinant p40^{AUF1}. RNP complexes were resolved on a native polyacrylamide gel by electrophoresis for 2 h in 0.5 $\times$ TBE buffer. (C,D) ARE RNA (2 nM) was incubated with 200 nM p40^{AUF1} (indicated by +), together with increasing amounts (from 5 to 50 nM) of EBER1 (C) or VAI RNA (D) as a competitor. All bandshift assays were conducted in the presence of 0.02 mg/mL tRNA as a nonspecific competitor.

confirmed interaction with EBER1 in vivo by using UV irradiation followed by stringent coimmunoprecipitation, indicating direct RNA-protein crosslinking. Similar analyses would be necessary to validate other hnRNP proteins as true interaction partners of EBER1.

If EBER1 binds and thus restrains AUF1 from interacting with ARE-containing mRNAs in EBV-infected cells, then the presence of EBER1 should increase the levels of ARE-containing mRNAs. In a recent study, Gregorovic et al. (2011) compared the transcript profiles of lymphoblastoid cell lines that were infected with either wild type or an EBV strain carrying a deletion for EBER1. Forty-one mRNAs were found to be significantly down-regulated in the cells lacking EBER1 compared with cells infected with wild-type virus (Gregorovic et al. 2011). A database search for AREs in the 41 down-regulated mRNAs (using ARED) (Bakheet et al. 2001, 2006) revealed that six of the 41 down-regulated transcripts contain an ARE (CHL1, CRIM1, KCNV1, NTRK2, IFNA6, and FUT4). We estimate the cellular abundance of the

p40 isoform of AUF1 to be about 6×10^6 molecules (data not shown), while EBER1 amounts to approximately 10^6 molecules in an EBV-infected cell (Lerner et al. 1981). Since multiple regions of EBER1 appear to interact with p40^{AUF1} (Fig. 4B), it is conceivable that EBER1 may largely sequester the available p40^{AUF1} molecules and thereby diminish their interaction with ARE-containing mRNAs in vivo. The fact that only a minor cohort of down-regulated mRNAs contained AREs (the TNF α mRNA level, e.g., was not affected by the presence of EBER1) (Supplemental Fig. S5) suggests that EBER1 possibly fulfills additional functions other than regulating the stability of ARE-containing mRNAs.

Recently, a novel function of AUF1 in suppression of senescence and telomere maintenance was reported (Pont et al. 2012). AUF1 was shown to bind to the promoter and thereby activate transcription of the telomerase catalytic subunit TERT (telomerase reverse transcriptase). Consistent with our observation that EBER1 can sequester AUF1,

we found that the mRNA level of TERT is reduced over twofold in EBER1-containing BJAB-B1 cells compared with isogenic BJAB cells (Supplemental Fig. S6). However, it was the p42 and p45 isoforms of AUF1 that were reported to be responsible for activation of TERT gene expression (Pont et al. 2012), whereas our *in vivo* interaction assay showed that these isoforms of AUF1 interact with EBER1 less well than the p40 isoform. Future studies, optimally in primary lymphocytes rather than immortalized cell lines, will be needed to resolve how interaction of the EBER1 RNP with AUF1 impacts telomere maintenance, ARE-containing mRNA stability, and other aspects of gene expression in EBV-associated lymphomas.

MATERIALS AND METHODS

EBER1 selection

BJAB cells, an EBV-negative Burkitt's lymphoma line, and BJAB-B1 cells, an EBV-converted BJAB cell line, were used (Menezes et al. 1975; Fresen et al. 1977). For isolating EBER1 with complementary oligonucleotides, a 2'-O-methyl modified RNA oligonucleotide complementary to EBER1 nucleotides 62–87 was used. The nucleotide at the 3'-end was not modified to allow oxidation and immobilization on adipic acid dihydrazide Agarose beads (Sigma), as described (Caputi et al. 1999; Borah et al. 2009). Total cell lysate from BJAB-B1 cells was used for isolation of EBER1 with complementary oligonucleotides.

The S1-aptamer (5'-accgaccagaatcatgcaagtgcgtaagatagtcgaggccgggg-3') or three bacteriophage MS2 coat protein binding sites (5'-cgtacaccatcagggtacgagctagcccatggcgtacaccatcagggtacgactagtagtctgacaccatcagggtacg-3'; MS2-hairpins are underlined) according to the method described by Zhou et al. (2002) were inserted into stem-loop IV of EBER1 (after nucleotide 106) by site-directed mutagenesis using Stratagene's QuikChange Site-Directed Mutagenesis Kit. Aptamer-containing EBER1 constructs were cloned into the lentiviral pTYF vector (Cao et al. 2008) and were expressed from the U6 promoter. Stable BJAB cell lines were generated by lentiviral transduction according to the method previously described (Cao et al. 2008). We lysed 10^7 BJAB cells expressing aptamer-containing EBER1 in 500 μ L lysis buffer (10 mM Hepes at pH 7.4, 150 mM KCl, 3 mM MgCl₂, 2 mM DTT, 0.5% NP-40, 10% glycerol) in the presence of protease inhibitor (1 mM PMSF; 1 $\times$ Protease Inhibitor Cocktail Set I; Calbiochem) and RNase inhibitor (80 units of RNasin; Promega). For MS2-mediated selection, 2 μ g of bacterially purified MS2-MBP fusion protein was added to the lysate and incubated for 3 h at 4°C before addition of amylose resin (NEB). The beads were washed five times with wash buffer (20 mM Tris at pH 7.4, 200 mM NaCl, 2 mM MgCl₂, 1 mM DTT) and eluted in wash buffer containing 10 mM maltose. SDS loading buffer was added to the eluate for Western blot analysis; for Northern blots, RNA was isolated by adding TRIzol (Invitrogen) to the eluate. Sequences of DNA oligonucleotides that were 5'-end-labeled with [γ -³²P]ATP for Northern blot analysis were 5'-cagctggtacttg accgaagac-3' (complementary to nucleotides 130–151 of EBER1) and 5'-attagagaatcctgacttgcaaatgtct-3' (complementary to nucleotides 106–134 of EBER2).

SILAC sample preparation and mass spectrometry analysis

Cells were cultured in RPMI-1640 media formulated for SILAC experiments (Pierce). BJAB cells stably expressing EBER1 or EBER1-MS2 hairpins were cultured in RPMI medium containing either naturally abundant ¹²C-, ¹⁴N-arginine and lysine (light medium) or heavy isotope-containing medium (¹³C-, ¹⁵N-arginine and lysine) for at least 10 doublings to ensure complete (>95%) endogenous labeling. We lysed 5×10^7 cells from each sample; equal amounts of proteins as determined by Bradford assay were mixed and subjected to MS2-mediated selection as described above. The eluted proteins were separated by SDS-PAGE, stained with colloidal Coomassie, and excised in five gel slices (for the MS2-selection where EBER1-MS2 expressing cells were cultured in heavy medium) or in seven gel slices (for the MS2-selection with reversed labeling). Proteins were prepared for mass spectrometry by cysteine reduction and alkylation, followed by overnight trypsin digestion at 37°C (Shevchenko et al. 2006). The tryptic peptides were desalted with C18 stage-tips (Agilent Technologies) and sequenced in a tandem mass spectrometer (LTQ-Orbitrap) at the Keck Proteomics Facility (Yale University).

The LTQ-Orbitrap raw data were analyzed using the software MaxQuant-Andromeda version 1.1.1.25 to identify the sequenced proteins and to determine their SILAC ratios (Cox et al. 2011). The SILAC label values were set to Arg0/Lys0 and Arg10/Lys6. Proteins with a single SILAC count were included in the quantification. MS/MS spectra were searched against the human International Protein Index (IPI) database (version 3.68). A false-discovery rate of 1% was selected using a randomized version of the human protein database. Other applied parameters were “filter labeled amino acids,” “re-quantify,” and “keep low scoring versions of identified peptides.”

Antibodies for Western blot analysis

Antibodies used for Western blot analyses were as follows: mouse anti-AUF1 at 1:2000 dilution (kind gift of Dr. Gideon Dreyfuss) (Pinol-Roma et al. 1988), rabbit anti-L22 at 1:5000 (Toczyski and Steitz 1993), mouse anti-La (1:500; kind gift of Dr. Ger Pruijn), mouse anti-Flag (1:2000; M2, Sigma), mouse anti-Myc (1:1000; 9E10, Santa Cruz), and mouse anti-nucleolin (1:2000; 4E2, Abcam).

In vivo UV crosslinking and immunoprecipitation

We washed 10^7 HEK293T or Raji cells with cold PBS, which were resuspended in 2.5 mL PBS and irradiated in a 10-cm dish on ice with 254-nm UV light at 800 mJ/cm². Cells were collected and lysed in 200 μ L of lysis buffer (50 mM Tris at pH 8.0, 1 mM EDTA, 1 mM DTT, 0.5% SDS) followed by heating for 5 min at 95°C and a short sonication pulse to increase solubility. Eight hundred microliters of correction buffer (62.5 mM Tris at pH 8.0, 187.5 mM NaCl, 1.75 mM EDTA, 1.25% NP-40, 0.625% sodium deoxycholate, 12.5% glycerol) was added together with protease inhibitor (1 mM PMSF; 1 $\times$ Protease Inhibitor Cocktail Set I; Calbiochem) and RNase inhibitor (80 units of RNasin; Promega). The lysate was precleared with protein A-Agarose beads for 1 h at 4°C, and antibodies (anti-L22 [Toczyski and Steitz 1993], a precipitating anti-AUF1 antibody [Dempsey et al. 1998]) were added

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