Nucleophosmin Contributes to the Transcriptional Activation Function of the Epstein-Barr Virus EBNA1 Protein

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The Epstein-Barr virus (EBV) EBNA1 protein plays important roles in latent infection, including transcriptional activation of EBV latency genes by binding to the family-of-repeats (FR) element. Through a proteomic approach, we previously identified an interaction between EBNA1 and the histone chaperone nucleophosmin. Here we show that the EBNA1-nucleophosmin interaction is direct and requires the Gly-Arg-rich sequences that contribute to transactivation. Additionally, nucleophosmin is recruited by EBNA1 to the FR element and is required for EBNA1-mediated transcriptional activation.

Epstein-Barr virus (EBV) is a gammaherpesvirus that infects greater than 95% of the world's adult population. The EBV infectious cycle is composed of lytic and latent phases, each characterized by the expression of specific viral genes. Upon primary infection, EBV establishes latency and the viral genome is circularized and maintained as nuclear episomes that are assembled into nucleosomes similar to cellular chromatin (1, 2). The EBV EBNA1 protein plays important roles in replicating and maintaining the EBV episomes, as well as in transactivating the expression of itself and other EBV latency genes important for cell immortalization (reviewed in reference 3). The transcriptional activation function of EBNA1 involves its binding to a cluster of 20 recognition sites in the family-of-repeats (FR) element of the latent origin of replication, *oriP*. The FR element also enhances reporter gene expression in an EBNA1-dependent manner.

Two EBNA1 regions have been shown to be critical for transcriptional activation; an N-terminal sequence mapping to amino acids 61 to 83 and a central Gly-Arg-rich region (amino acids 325 to 376) (4–6). The amino acid 61-to-83 region mediates an interaction with Brd4, an acetylated-histone-binding protein known to contribute to the transcriptional activation of both cellular and viral genes (7–10). The central Gly-Arg region contributes to interactions with the histone chaperones NAP1 and TAF-I (11, 12).

Recently, we conducted a series of EBNA1 proteomic experiments in the context of EBV-infected cells and found that, in addition to NAP1 and TAF-I, EBNA1 also associates with the histone chaperone nucleophosmin (NPM1; also called B23) (13). The EBNA1-NPM1 interaction was confirmed by coimmunoprecipitation and was unaffected by RNase treatment, showing that it is not RNA mediated (13). NPM1 has many cellular functions, including, ribosome biogenesis, histone assembly, regulation of DNA integrity, cell proliferation, and regulation of the tumor suppressors p53 and ARF (14–18). In addition, NPM1 has been shown to contribute to the transcription of both cellular and viral genes and to functionally interact with several viral proteins, including EBV EBNA2, HIV-1 Rev and Tat, and the hepatitis virus δ antigen (19–22). The NPM1-EBNA2 interaction was shown to be important for EBNA2-dependent transcription and transformation in lymphoblastoid cells (22).

To investigate the significance of the EBNA1-NPM1 interaction, we first asked whether this interaction was direct. To do this, we purified His-tagged EBNA1 from insect cells as previously described (11) and purified STREP-tagged NPM1 from insect cells

FIG 1 Nucleophosmin directly interacts with EBNA1. One micromole of purified, His-tagged EBNA1 (lanes L1) was incubated with 20 μ l of nickel resin, and the unbound His-EBNA1 (lanes F1) was subsequently collected. Three micromoles of purified, STREP-tagged NPM1 (lanes L2) was added to nickel resin alone or resin containing His-EBNA1 as indicated, and the unbound STREP-NPM1 was then collected (lanes F2). Complexes were eluted with buffer containing 250 mM imidazole (lanes E).

by avidin affinity chromatography. *In vitro* pulldown assays were performed by immobilization of His-EBNA1 on nickel resin, followed by incubation with excess STREP-NPM1 in binding buffer (50 mM Tris [pH 7.9], 250 mM NaCl, 10% glycerol, 20 mM imidazole, 0.1 mM dithiothreitol, protease inhibitor cocktail) and then four washes with binding buffer prior to elution of the complexes with 250 mM imidazole. As shown in Fig. 1, STREP-NPM1 coeluted with EBNA1, whereas there was no detectable binding of STREP-NPM1 to the resin in the absence of EBNA1. Interestingly we did not find an interaction between EBNA1 and the NPM1 protein purified from *Escherichia coli*, suggesting that posttranslational modifications of NPM1 are required for its interaction with EBNA1 (data not shown).

Since NPM1 has known roles in transcriptional activation, we tested whether NPM1 plays a role in EBNA1-mediated transcriptional activation by using a chloramphenicol acetyltransferase (CAT) reporter assay. In this assay, the expression of CAT is under the control of the FR enhancer element, which requires EBNA1

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FIG 2 Effect of NPM1 depletion on transcriptional activation by EBNA1. After transfection with AllStars negative-control siRNA (minus siNPM1) or NPM1-targeted siRNA (plus siNPM1), cells were transfected with an EBNA1 expression plasmid (plus EBNA1) or an empty plasmid (minus EBNA1), an FR-CAT reporter plasmid that is EBNA1 dependent, and a SEAP reporter plasmid that is EBNA1 independent. Effects on CAT expression (left bar graph) and SEAP expression (middle bar graph) were determined separately. In the right bar graph, CAT levels were normalized to SEAP levels to account for any nonspecific transcriptional effects. Expression of EBNA1 and the degree of NPM1 depletion are shown in the Western blot on the left.

binding for CAT expression (12). The CAT reporter plasmid was cotransfected with an EBNA1 expression vector or an empty vector. To monitor any general effects on transcription that are independent of EBNA1, a third plasmid containing a constitutively active secreted embryonic alkaline phosphatase (SEAP) reporter was also included in the cotransfection. In order to determine if NPM1 has an effect on EBNA1-dependent transcription, CNE2Z cells (EBV-negative nasopharyngeal carcinoma) were transfected with a small interfering RNA (siRNA) targeting NPM1 or a negative-control siRNA (AllStars; Qiagen, 1027281) and transfected 2 days later with the three plasmids. Seventy-two hours later, cells were lysed and assays for CAT and SEAP were performed as previously described (12). As expected, in control siRNA-treated samples, the presence of EBNA1 resulted in a 5-fold increase in CAT expression over that in the absence of EBNA1 (Fig. 2). However, when NPM1 was downregulated with siRNA, CAT expression in the presence of EBNA1 was reduced to the background levels seen in the absence of EBNA1, despite the fact that EBNA1 levels were unaffected by NPM1 depletion. In contrast, NPM1 depletion had no effect on the expression of SEAP from the EBNA1-independent reporter, indicating that NPM1 specifically contributed to EBNA1-mediated transcriptional activation.

If NPM1 contributes directly to EBNA1-mediated transcriptional activation, then we would expect to find it associated with EBNA1 at the *oriP* FR element in EBV genomes. To examine this possibility, we performed chromatin immunoprecipitation

(ChIP) experiments with EBV-positive gastric carcinoma cells, AGS-EBV (23). Cells were harvested, fixed, and lysed, and DNA was sheared to 0.5 to 1 kb as previously described (12). Immunoprecipitations were performed with antibody against NPM1 (sc-32256; Santa Cruz) or a nonspecific IgG (sc-205; Santa Cruz) as a negative control, and the recovered DNA fragments were quantified by quantitative PCR (qPCR) with primer sets specific to the FR element, Qp promoter, or LMP1 promoter region along with SYBR green qPCR SuperMix (Bio-Rad) in a Rotorgene qPCR system. qPCR was also performed with samples directly after the shearing step (input), and the values obtained with ChIP samples were normalized to the input for each primer set. The results of three independent experiments showed that NPM1 could be detected in the FR element ($P < 0.05$) but not in the Qp and LMP1 promoter regions of the EBV genome (Fig. 3A). Since EBNA1 also binds to the dyad symmetry (DS) element of *oriP* (as part of its DNA replication function), recovery of the DS element was also examined in the ChIP experiments (Fig. 3A) and NPM1 was also consistently detected at this element.

To determine if the association of NPM1 with the *oriP* elements depended on EBNA1, EBNA1 was silenced in AGS-EBV cells with siRNA as previously described (24) and ChIP with NPM1 antibody was performed. The EBNA1 siRNA treatment decreased the recovery of NPM1 at both the DS and FR elements 2- to 3-fold relative to that after treatment with AllStars negative-control siRNA but did not affect total levels of NPM1 (Fig. 3B).

FIG 3 Localization of NPM1 to *oriP* elements by ChIP. (A) ChIP experiments were performed with AGS-EBV cells and antibodies against NPM1 or nonspecific mouse IgG. Recovered DNA fragments were quantified by real-time PCR with primer sets for the *oriP* DS and FR elements and the LMP1 and Qp promoter regions. The amplification signals were normalized to those from the same cell lysates prior to immunoprecipitation with the same primer pairs. Signals from NPM1 antibody samples were expressed relative to those for the control IgG samples, which were set to 1. The results shown are from three independent experiments, with PCR quantification performed in triplicate for each experiment. *, $P < 0.05$. (B) AGS-EBV cells were treated with siRNA against EBNA1 (siE) or AllStars negative-control siRNA (siC), and ChIP assays were performed for NPM1 as in panel A with DS and FR primer sets. The effects of siRNA treatments on EBNA1 and NPM1 levels are shown in the Western blot assay.

FIG 4 Coimmunoprecipitation of mutant EBNA1 proteins with NPM1. (A) Schematic representation of EBNA1 showing the locations of the Gly-Arg-rich regions, the 61-to-83 transactivation sequence, the DNA binding domain, and the Gly-Ala repeat as found in the B95-8 version of EBNA1. (B) 293T cells were transfected with *oriP* plasmids containing either WT EBNA1 or the indicated mutant EBNA1 proteins. At 72 h posttransfection, NPM1 was immunoprecipitated (IP) from cell lysates (with antibody sc-32256) and recovered proteins were detected by Western blotting with antibody specific for EBNA1 (R4) and NPM1 (sc-6013). One-hundredth of the starting lysate was also analyzed directly by Western blotting to determine EBNA1 expression levels (Input). (C) Quantification of coimmunoprecipitated EBNA1 normalized to the input. Mutant EBNA1 levels are shown relative to the result obtained with WT EBNA1, which was set to 1. Averages of three independent experiments are shown with standard deviations. **, $P < 0.01$; ***, $P < 0.001$.

Therefore, the association of NPM1 with *oriP* requires EBNA1, suggesting that NPM1 associates with the *oriP* elements by interacting with EBNA1. Note that while the association of NPM1 with the DS element could mean that it also contributes to EBNA1-mediated DNA replication from this element, the requirement of NPM1 for replication from *oriP* could not be tested. This is because even partial downregulation of NPM1 with siRNA was found to inhibit cell proliferation (in two different cell lines that were tested), which is required for replication from *oriP*.

Finally, we mapped the region of EBNA1 that is important for the NPM1 interaction by coimmunoprecipitation assays with a

series of mutant EBNA1 proteins that we have previously characterized, including mutant EBNA1 proteins specifically lacking either of the transactivation sequences (amino acids 61 to 83 or 329 to 376). 293T cells were transfected with plasmids expressing EBNA1 or deletion mutant EBNA1 proteins and endogenous NPM1 was immunoprecipitated (Fig. 4). As expected, wild-type (WT) EBNA1 was efficiently recovered with NPM1. $\Delta 61-83$ mutant EBNA1 was also recovered efficiently, indicating that the amino acid 61 to 83 transactivation region is not responsible for the interaction with NPM1. However, in three independent experiments, the recovery of EBNA1 was decreased when the Gly-Arg-rich transactivation domain was deleted ($\Delta 329-376$), as well as when the N-terminal Gly-Arg-rich region was deleted ($\Delta 34-52$). Deletion of both the Gly-Arg-rich regions ($\Delta 34-52$ $\Delta 329-376$) abrogated detectable binding to NPM1, further emphasizing their importance for the NPM1 interaction. This is similar to EBNA1 interactions with the NAP1 and TAF-1 histone chaperones, which also involve the Gly-Arg-rich EBNA1 regions (11, 12). NPM1, NAP1, and TAF-I all contain acidic regions that might be responsible for these interactions. Our present and previous results combined suggest that EBNA1 can interact with any of these histone chaperones and use them to activate transcription. However, EBNA1-mediated transcriptional activation was more dramatically affected by NPM1 depletion than was previously observed for NAP1 or TAF-I depletion (12), suggesting either that the EBNA1-NPM1 interaction is the most important for transactivation or that NPM1 is more limiting.

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