A potential NES of the Epstein-Barr virus nuclear antigen 1 (EBNA1) does not confer shuttling

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Abstract The Epstein-Barr virus nuclear antigen 1 (EBNA1) is a multifunctional protein involved in the replication and maintenance of the viral episome. We identified a potential Rev-like nuclear export signal (NES) which, however, does not confer the export of EBNA1. In the yeast two-hybrid system EBNA1 does not bind to the nuclear exporter Crm1p. In spite of the RNA-binding ability of EBNA1 and its structural homologies to RNA binding proteins like hnRNP U and/or A1, EBNA1 does not shuttle to the cytoplasm in heterokaryon analysis. We propose the function of the RNA binding of EBNA1 in retaining RNAs to the nucleus.

Key words: EBNA1; Nuclear export; Heterokaryon assay

1. Introduction

The Epstein-Barr virus (EBV) is the causative agent of infectious mononucleosis and is furthermore implicated in the pathogenesis of several human malignancies (reviewed in [1]). EBNA1 is the only latent protein which is expressed in all types of latency found in human EBV-positive malignancies.

EBNA1 is a multifunctional phosphoprotein [2] with a variety of properties including sequence-specific binding to DNA [3] [4], formation of homodimers [5], nuclear localization [6] and stimulation of cellular gene expression [7]. EBNA1 binds to the viral origin of replication (oriP) [8] [9] and is the only virus protein necessary and sufficient to replicate and maintain the EBV genome in the infected cell [10]. Recently, it was shown that one of the roles in oriP replication mediated by EBNA1 is to prevent the degradation of newly synthesized oriP plasmids [11]. The initiation of the DNA synthesis at oriP can be induced by cellular factors but probably facilitated by EBNA1. So far, there is no helicase or ATPase activity known for EBNA1.

Using the yeast two-hybrid system we identified the first EBNA1 associated protein, the nuclear transporter karyopherin α2/Rch1 [12]. We demonstrated that the binding to karyopherin α2 was mediated through a C-terminal region of EBNA1 encompassing the nuclear localization signal (NLS). Experiments analyzing the cellular distribution of EBNA1 revealed that a small portion of the protein could be found in the soluble cytoplasmic fraction [12]. Immunohistochemical staining of EBNA1 positive tumor cells also showed a weak cytoplasmic staining of EBNA1 [13,14].

EBNA1 shares significant structural similarity to the RNA-binding domains of the U1A spliceosomal protein and heterogeneous ribonucleoprotein (hnRNP) [15]. RNA-binding proteins, like hnRNPA1, were shown to shuttle between nucleus and cytoplasm, suggesting that they may be involved in nucleocytoplasmic trafficking of mRNA [16]. Other hnRNP proteins, like hnRNP U are restricted to the nucleus implicating that one of their function may be retaining incompletely processed pre-mRNA in the nucleus. These proteins are to be removed from the RNA before translocation to the cytoplasm. One of the best characterized nuclear export mechanisms is the Rev-like pathway based on a small transferable leucine rich nuclear export signal (NES) which confers binding to the nuclear export factor Crm1p [17]. In an energy-dependent mechanism proteins bearing a NES are transported to the cytoplasm. A potential Rev-like NES is located in the C-terminal region of EBNA1 (aa 533–540) (see Fig. 1). Furthermore, EBNA1 was found as a component of an RNA complex and it could be demonstrated that EBNA1 binds to the EBV-encoded RNA-polymerase III transcript, EBER 1, as well as to exon sequences derived from its own RNA [18]. Since EBNA1 encompasses a potential NES, binds to RNA and is partially found in the cytoplasm, we speculated that the cytoplasmic form of EBNA1 might be involved in the translocation of RNA from the cell nucleus to the cytoplasm. Our aim was to investigate whether the nuclear form of EBNA1 indeed shuttles to the cytoplasm. We found by heterokaryon assays, however, that EBNA1 is retained in the nucleus. Also, no binding of EBNA1 to the export factor Crm1p in a yeast two-hybrid assay was detectable. We propose that the function of the RNA binding ability of EBNA1 may lie in retaining incompletely processed pre-mRNA in the nucleus, like its own RNA transcribed from the Fp promoter.

2. Materials and methods

2.1. Plasmid construction

The plasmid pEGFP-EBNA1 containing a truncated form of EBNA1 was constructed as follows: the EBNA1 gene with only 5 GGA repeats, normally located between aa 90 and 328, from the expression vector p1553 [11] was PCR amplified and cloned in frame into the *Eco*RI, *Sal*I digested pEGFP-C1 vector (Clontech). The vector p1553 was a generous gift of B. Sugden (Madison, WI, USA). The primers used were: 5′-GCGAATTCCATGTCTGACGAG-3′ and 5′-CACGCCAGCTGTCACTCCTGCCCCTTCTCACC-3′.

The plasmids encoding adenovirus E4orf6 as well as E4orf6, mutated within the potential NES coding region, were kindly supplied by M. Dobbelstein (Marburg, Germany).

2.2. Cells and antibodies

HeLa cells and murine BALB/c 3T3 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and were subcultured routinely once per week. The rat monoclonal anti-

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body (MAb) 1H4 directed against EBNA1 has been described [14]. The mouse MAb RSA3 [19] directed against adenovirus E4orf6 was a generous gift from M. Dobbelstein (Marburg, Germany).

2.3. Protein shuttling assay

Shuttling assays were performed using a heterokaryon assay as described by Pinol-Roma and Dreyfuss [20]. HeLa cells were transfected with various expression plasmids using the Superfect Transfection reagent (Qiagen). 24–30 h after transfection, the cells were seeded on a 3.5 cm tissue culture dish containing a glass cover slip together with 1×10^6 BALB/c 3T3 cells in culture medium containing cycloheximide (50 μg/ml). After 2–3 h the cells were covered with a solution of 50% (w/v) polyethylene glycol 8000 (Sigma) in PBS for 2 min at 37°C to induce cell fusion. Subsequently, the coverslips were transferred back to DMEM/10% FCS containing cycloheximide and further incubated for 2 h, 4 h or 6 h at 37°C. The cells were then fixed with 4% paraformaldehyde in PBS for 15 min, followed by permeabilization with 0.2% Triton X-100 in PBS at 4°C. The cells were analyzed by immunofluorescence using primary antibodies RSA3 [19] at a 1:10 dilution or 1H4, anti-EBNA1, at a 1:10 dilution, for 1 h, washed three times for 5 min in PBS. Secondary antibodies (FITC-conjugated goat anti-mouse IgG2a or anti-rat IgG, diluted 1:500) were added for 30 min, followed by three washes as above and subsequently mounted in DAPI solution (Sigma) for 30 min. The cells transfected with the pEGFP constructs were directly treated with the DAPI solution after fixation.

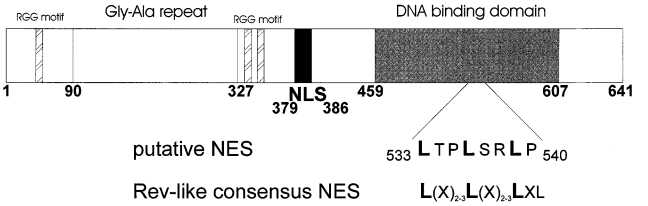

Fig. 1. Diagram of the 641 aa EBNA1 protein. The RGG motifs, the position of the Gly-Ala repeat, the NLS and the DNA binding domain are indicated. The sequences of the potential NES and of the consensus Rev-like NES are shown.

3. Results and discussion

3.1. Identification of a potential Rev-like NES in the C-terminal domain of EBNA1

Previous studies from Ambinder et al. [6] had identified a classical nuclear localization sequence (NLS) between aa 379 and 386 of the EBNA1 protein. Consistent with these earlier results we could show that indeed this NLS mediates the transport of EBNA1 from the cytoplasm to the nucleus [12].

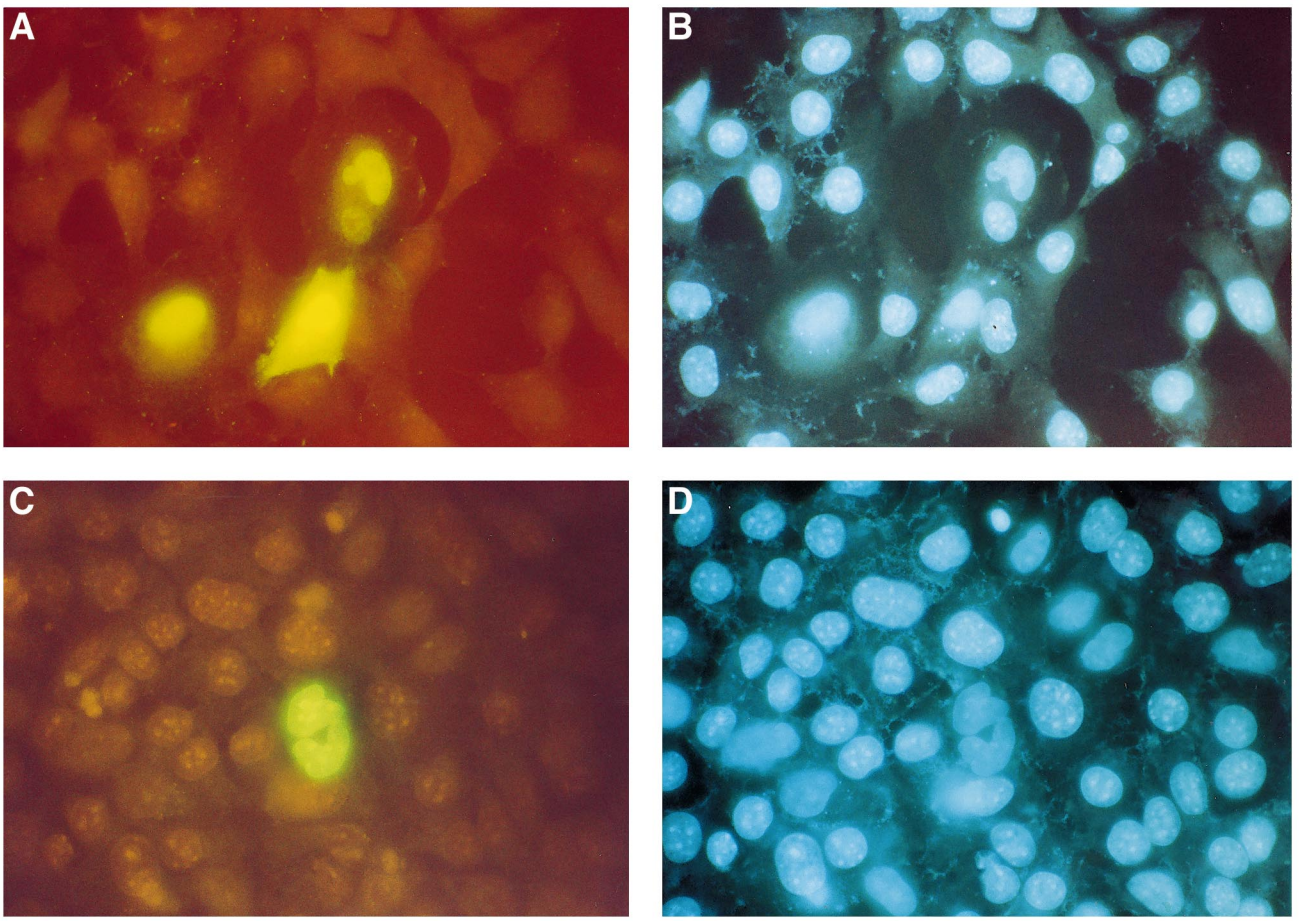

Fig. 2. EBNA1 does not traffic between nuclei of heterokaryons. HeLa cells were transfected with expression plasmids for E4orf6 (A,B), and GFP-EBNA1 (C,D). After heterokaryon formation with murine cells, the cells were incubated for 2 h at 37°C. Then, the heterokaryons were fixed and stained for E4orf6 (A) and for EBNA1 (C) using the monoclonal antibodies RSA3 and 1H4, respectively. This was followed by an FITC-conjugated secondary antibody for rat IgG. Subsequently, the cells were stained with a DAPI solution 1:25 000 (Sigma) for distinguishing the nuclei of the different species (B,D). In contrast to the human nuclei, the nuclei of the murine BALB/c 3T3 showed specific bright speckles. The images displayed are examples of the results consistently observed in >100 fused cells viewed over three independent cell fusion experiments.

EBNA1 is restricted to bright speckles within the nucleus and also thinly distributed in the whole nucleus, suggesting that the major part of EBNA1 is probably confined to replication origins [13,14]. In contrast, cellular fractionation studies [12] as well as immunohistochemical staining of tumor cells revealed that also a minor part of EBNA1 can be found in the cytoplasm, indicating that EBNA1 might be exported out of the nucleus. By analysis of the EBNA1 aa sequence we found that the carboxy-terminal sequence between aa 533 and aa 540 (**L**^{533}**TP**L**SR**L**P**) shares significant similarity with the consensus Rev-like NES sequence (reviewed in [21]) (Fig. 1). This nuclear export sequence (NES) consists of several leucine residues with uneven spacing in-between. The consensus pattern L(X)2–3L(X)2–3LXL was first described for the HIV-1 Rev and cellular protein kinase inhibitor (PKI) proteins [22,23], and is targeted by Crm1p, a member of the karyopherin β family [24–27]. These observations suggested that the presence of EBNA1 in the cytoplasm might be a consequence of Crm1p-dependent export of EBNA1. Therefore, we tested the interaction between Crm1p and EBNA1 in the yeast two-hybrid system. In these experiments different fusion proteins of EBNA1 containing this potential NES did not show an interaction with Crm1p in the yeast two-hybrid system (data not shown). This implies that EBNA1, if exported out of the nucleus, does not use the Rev-like pathway.

For the hnRNP A1 it was shown that inhibition of RNA transcription by actinomycin D, inhibitor of the RNA polymerase II, resulted in retention of the protein in the cytoplasm [20]. We tested whether the nuclear localization of EBNA1 is also dependent on RNA polymerase II transcription. For this purpose, we exposed GFP-EBNA1 transfected HeLa cells with actinomycin D. After treatment of the GFP-EBNA1 transfected cells for 2, 4 or 8 h with actinomycin D, we observed no change in the EBNA1 staining, i.e. EBNA1 was still predominantly found in the nucleus (data not shown). This suggested that the cytoplasmic localization of EBNA1 neither depends on RNA export nor on impaired nuclear import caused by inhibition of RNA transcription.

3.2. EBNA1 does not shuttle between nucleus and cytoplasm

Since EBNA1 in addition to its potential NES, shows sequence similarities to heterogeneous RNA-binding proteins like hnRNP U and hnRNP A1, we asked whether the cytoplasmic localization of EBNA1 is due to the shuttling of the protein between nucleus and cytoplasm. The sequence of EBNA1 shows a strong homology with the heterogeneous nuclear protein, hnRNP U (a member of the hnRNP particles, controlling post-transcriptional processes) in regard to RGG consensus RNA binding motifs [17]. Furthermore, EBNA1 possesses structural homologies to the heterogeneous ribonucleoprotein hnRNP A1, which is involved in mRNA export and continuously shuttles between nucleus and cytoplasm [13]. Nascent pre-mRNAs associate with the abundant heterogeneous nuclear RNP (hnRNP) proteins and remain associated with them as long as they reside in the nucleus. The hnRNP proteins can be divided into two groups according to their nucleocytoplasmic transport properties. One group is completely restricted to the nucleus in interphase cells, whereas the other group, although primarily nuclear at steady state, shuttles between the nucleus and the cytoplasm. To assay for nucleocytoplasmic shuttling, heterokaryons of E4orf6 and pEGFP-EBNA1 transfected HeLa cells, respectively, with murine BALB/c 3T3 cells were produced using polyethylene glycol (see Section 2). The cells were fixed and stained, 2, 4 or 6 h after the fusion followed by a stain with a DAPI solution thus distinguishing the nuclei of the different species from each other. Nucleocytoplasmic shuttling was indicated by the detection of adenovirus E4orf6 in the murine nuclei, which served as a positive control (Fig. 2A,B). In contrast to this, EBNA1 was only found in the originally transfected human HeLa cell nuclei (Fig. 2C,D), indicating that in spite of the structural similarities between EBNA1 and hnRNP A1, EBNA1 does not shuttle between nucleus and cytoplasm.

The function of the RNA binding by the RGG motifs of EBNA1 seems not to lie within the transport of viral or cellular RNA into the cytoplasm. It was shown that the non-shuttling group of hnRNP are restricted to the nucleus not because they lack a NES, but because they bear a nuclear retention sequence (NRS) that is capable of overriding an NES [28]. It was demonstrated by Snudden et al. [17] that EBNA1 could bind to exon sequences derived from its own RNA, transcribed from the Fp promoter. In regard to this, it could be possible that the RNA binding activity of EBNA1 leads to regulation of the *Bam*HI W transcripts. It will be of great interest to further analyze the function of the RNA-binding activity of EBNA1 in regard to its possible role in retaining viral or cellular RNAs within the nucleus.

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