Epstein–Barr virus nuclear antigen 5 interacts with HAX-1, a possible component of the B-cell receptor signalling pathway

Martin Dufva, Maria Olsson and Lars Rymo

Using a yeast two-hybrid screen of a B-cell cDNA library with an Epstein–Barr nuclear antigen 5 (EBNA5) molecule containing seven repeats of the W1W2 domain as bait, we have isolated the EBNA5-interacting protein HAX-1. HAX-1 has previously been shown to associate with HS1, a protein specifically expressed in cells of the haematopoietic lineage, and is thought to be involved in signal transduction in B-cells. Immunofluorescence experiments showed that HAX-1 co-localized with the hsp60 protein that is associated with the mitochondria in the cell cytoplasm. Pull down experiments with a fusion protein between glutathione S-transferase and the seven copy repeat EBNA5 synthesized in bacteria and in yeast cells confirmed that HAX-1 can interact with EBNA5 in vitro. Conventionally, EBNA5 is regarded as a nuclear protein. However, we show here that the smallest EBNA5 species, composed of the unique Y domain and only one copy of the W1W2 repeat domain, like HAX-1, co-localizes with the mitochondrial hsp60 protein in the B-cell cytoplasm. Furthermore, immunoprecipitation experiments demonstrate that the single repeat EBNA5 associates with HAX-1 in transfected B-lymphoblastoid cells.

Introduction

Epstein–Barr virus (EBV) is a ubiquitous herpesvirus in humans that can infect, establish latency and induce proliferation in B-lymphocytes. The EBV genome contains more than 80 genes that encode structural and regulatory proteins. A subset of these genes has been shown to be required for the efficient differentiation of EBV-infected resting B-cells into proliferating B-lymphoblasts (immortalization). They include the nuclear proteins EBNA1, EBNA2, EBNA3 (also designated EBNA3A), EBNA5 (also designated EBNA-LP) and EBNA6 (also designated EBNA3C), and the membrane protein LMP1 (reviewed by Kieff, 1996). In the EBV-infected lymphoblastoid cell all of the EBNA genes have been implicated in the control of gene expression. The EBNA2- and EBNA5-encoding sequences are promoter-proximal in the EBNA transcription unit and the proteins appear within 6 h after infection (Alfieri et al., 1991; Dillner & Kallin, 1988). The EBNA5 mRNA is formed by an initial splice between a promoter-proximal exon and the first exon of the repeat (W1L), which creates a translation initiation codon and the EBNA5-encoding open reading frame. Then mRNAs carrying different numbers of W1/W2 exon pairs are generated by alternative splicing between the repeated W1 and W2 exons and the unique Y1 and Y2 exons. These are translated into polypeptides containing a variable number of copies of a 66 amino acid repeat domain and a 45 amino acid unique domain. Thus, in the infected B-cell, EBNA5 often appears in SDS–PAGE/immunoblotting experiments as a ladder of proteins in a size range from 20 to 78 kDa (Finke et al., 1987).

In spite of considerable efforts, our knowledge about the biochemical functions of EBNA5 and its role in the immortalization process is still fragmentary. The amount of EBNA5 observed during infection rises to high levels in the first 3 to 4 days of infection and then decreases to the levels found in established lymphoblastoid cell lines (LCLs) (Szekely et al., 1995b). EBNA5 antigens appear very early and are at first diffusely distributed throughout the nucleoplasm (Szekely et al., 1995a, 1996). After a few days the EBNA5 immunostaining condenses into discrete foci which coincide with nuclear bodies known as ND10 domains or promyelocytic leukaemia-associated protein (PML) oncogenic domains (PODs). So far about 20 POD-associated proteins have been identified, including the PML protein, the retinoblastoma protein (Rb), the heat shock protein 70 (hsp70) and the transcriptional

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coactivator CREB-binding protein. Evidence is accumulating which suggests that PODs are involved in both transcription regulation, cell-cycle regulation and regulation of cell death (for overviews see Hess & Korsmeyer, 1998; Matera, 1999). EBNA5 has been shown to co-localize with hsp70 in vivo (Szekely et al., 1995a), and studies in vitro have demonstrated a physical interaction between the proteins (Kitay & Rowe, 1996b; Mannick et al., 1995). The implication of this interaction for the biology of the virus remains to be elucidated. EBNA5 has also been shown to bind to the Rb and p53 proteins (Szekely et al., 1993). However, it has so far not been possible to reveal any effect of EBNA5 on Rb or p53 in their function as regulators of transcription (Inman & Farrell, 1995). EBNA5 has also been shown to associate with the nuclear matrix and it appears that EBNA5 increases the affinity of EBNA6 to this structure (Cludts & Farrell, 1998; Szekely et al., 1995a).

EBNA5 might be involved in the regulation of the cell cycle. Cyclin D2 expression was upregulated in resting B-lymphocytes in response to cotransfection with EBNA2 and EBNA5 expression vectors, making the cells leave the G0 phase and enter the cell cycle (Sinclair et al., 1994). Moreover, the phosphorylation status of EBNA5 changes during the cell cycle, as shown by an increased number of phosphorylated serine residues in EBNA5 during the G2 phase, reaching a maximum of about 50% at the G2/M boundary (Kitay & Rowe, 1996a). Direct evidence for the role of EBNA5 in transcription regulation was lent by recent studies showing that EBNA5 cooperates with EBNA2 to activate the expression of LMP1 (Harada & Kieff, 1997; Nitsche et al., 1997). EBNA5 enhanced the EBNA2-mediated induction of endogenous LMP1 expression in Eli-BL cells and was absolutely required for the induction of LMP1 expression in Akata-BL cells (Nitsche et al., 1997). Notably, the Cp promoter was not induced by the same procedures that activated LMP1 expression (Nitsche et al., 1997). In a transient transfection study with reporter plasmids, it was shown that EBNA5 potentiated EBNA2 activation of the LMP1 promoter in BJAB cells (Harada & Kieff, 1997). EBNA5 also stimulated EBNA2 activation of reporter plasmids that contained either a multimer of a regulatory region from the Cp promoter or a synthetic DNA fragment containing five RBP-Jκ-binding sites.

The general objective of the present study was to broaden our understanding of the role of EBNA5 in transcription regulation. To identify EBNA5-interacting proteins, we performed a yeast two-hybrid screen of a B-cell cDNA library with EBNA5 as bait. In this paper we characterize the interaction between EBNA5 and the HS1-associated protein X-1 (HAX-1), a cytoplasmic protein that might be involved in signal transduction in B-cells. We also show that the smallest EBNA5 species, composed of the unique Y domain and only one copy of the W1W4 repeat domain, in contrast to larger EBNA5 species, is present in the cytoplasm but not in the nucleus of transfected B-lymphoid cells and associates with HAX-1 in the cells.
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For two-hybrid screening we used a fusion protein bait consisting of the yeast GAL4 DNA-binding domain (BD; amino acids 1–147) and the EBNA5 protein expressed from a yeast shuttle vector. The yeast strain Y190 was transformed with pAS2–EBNA5 DNA. Yeast cells expressing the bait were transformed with a cDNA library constructed from EBV-transformed B-cells. About $3\times10^6$ transformants were screened for the activation of the reporter lacZ and 70 positive clones were isolated and sequenced. A database search using the BLAST program showed that the DNA sequence of 14 clones was identical to the sequence of HAX-1 cDNA. Eleven of the clones represented full-length cDNAs (HAX-1 24.1) and three of the clones shorter molecules (HAX-1 67.2) lacking nucleotides corresponding to the first 48 amino acid residues of the N terminus of the protein. The relative levels of interaction of HAX-1 24.1 and HAX-1 67.2 with EBNA5 were determined by quantifying the levels of expression of the lacZ reporter gene. Cells expressing the bait protein and HAX-1 tagged with the GAL4 AD induced significant levels of $\beta$-galactosidase compared with the control cells expressing the BD fusion protein and AD vector (Table 1). The results also indicated that

Table 1. Relative level of interaction between EBNA5 and HAX-1

<table>
<thead>
<tr>
<th>Expression vectors*</th>
<th>Filter assay†</th>
<th>Relative $\beta$-Gal activity</th>
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<tbody>
<tr>
<td>pCL1</td>
<td>B</td>
<td>3700±1100</td>
</tr>
<tr>
<td>pAS2</td>
<td>W</td>
<td>0±0.3</td>
</tr>
<tr>
<td>pAS2–EBNA5</td>
<td>W</td>
<td>0±0.1</td>
</tr>
<tr>
<td>pAS2–EBNA5 + pACT2-HAX-1(24.1)</td>
<td>W</td>
<td>1±0±0.3</td>
</tr>
<tr>
<td>pAS2–EBNA5 + pACT2-HAX-1(67.2)</td>
<td>W</td>
<td>1±0±0.4</td>
</tr>
<tr>
<td>pAS2–EBNA5 + pACT2-HAX-1(24.1)</td>
<td>B</td>
<td>38±7</td>
</tr>
<tr>
<td>pAS2–EBNA5 + pACT2-HAX-1(67.2)</td>
<td>B</td>
<td>140±23</td>
</tr>
<tr>
<td>pAS2–EBNA5 + pACT2</td>
<td>W</td>
<td>1±4±0.3</td>
</tr>
</tbody>
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* pCL1 encodes the full-length GAL4 molecule. pACT2 vectors encode GAL4-AD (activation domain) fusion proteins and pAS2.1 vectors encode GAL4-BD (binding domain) fusion proteins.
† Results of the X-Gal colony lift assay are indicated with B for blue colonies and W for white.

Results and Discussion

For coimmunoprecipitation experiments, cell extracts were prepared by resuspending $2\times10^7$ cells cotransfected with the HAX-1 expression vector pME18s–HAX-1 and either the pCI (Promega), pCI-E5(W01W2Y) or pCI-EBNA5 plasmids in lysis buffer [150 mM NaCl, 50 mM Tris–HCl, pH 7.4, 150 mM MgCl2, 0.1% Nonidet P-40 and centrifugation at 14000 $g$ for 10 min. All extracts were precleared by incubation with glutathione-conjugated Sepharose beads (Pharmacia) prior to the GST pull down experiments.

For immunoprecipitation experiments, cell extracts were prepared by resuspending $2\times10^7$ cells cotransfected with the HAX-1 expression vector pME18s–HAX-1 and either the pCI (Promega), pCI-E5(W01W2Y) or pCI-EBNA5 plasmids in lysis buffer [150 mM NaCl, 50 mM Tris–HCl, pH 7.4, 150 mM MgCl2, 0.1% Nonidet P-40, 0.5 mM pefabloc (Roche), 5 $\mu$g/ml antipain (Roche), 5 $\mu$g/ml leupeptin (Roche) and 5 $\mu$g/ml aprotinin (Roche)]. The cell suspension was kept on ice for 15 min, sonicated three times for 10s at 10 $\mu$m, and cleared by centrifugation for 30 min at 4°C and 18000 $g$. The supernatant was incubated with or without the JF186 anti-EBNA5 antibody for 16 h at 4°C. The immune complex was collected using protein G-conjugated Sepharose beads (Pharmacia Biotech) and washed extensively. The adsorbed proteins were eluted by boiling in SDS sample buffer and analysed under denaturing conditions by electrophoresis in 10% polyacrylamide gels (SDS–PAGE) and immunoblotting. HAX-1 and EBNA5 were visualized on the blots by alkaline phosphatase (AP)-conjugated rabbit anti-mouse antibody (Dako) and a nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (BCIP/NBT) colorimetric AP reaction (Promega).

Antibodies and immunostaining. GAL4 activation domain (AD) fusion protein was detected with anti-GAL4-AD antibody (Clontech). EBNA5 was detected with the mouse monoclonal anti-EBNA5 antibody JF186 (Dillner et al., 1996) obtained from Dr M. Masucci, MTC, Karolinska Institute, Stockholm. Anti-HAX-1 antibody was purchased from Transduction Laboratories. Hsp60 was detected by immunostaining using the goat anti-hsp60 antibody (Santa Cruz Biotechnology) as the primary antibody and rhodamine-conjugated donkey anti-goat IgG antibody (Santa Cruz Biotechnology) as the secondary antibody. E5(W01W2Y) and HAX-1 were detected by immunostaining using JF186 anti-EBNA5 and anti-HAX-1 antibodies, respectively, as primary antibodies, and FITC-conjugated rabbit anti-mouse IgG antibodies (Dako) in a first step and FITC-conjugated swine anti-rabbit IgG antibodies (Dako) in a second step as secondary antibodies.

Preparation of protein-containing cell extracts. Yeast GST–EBNA5 was expressed by pYEX–EBNA5 in the yeast Y150 strain using established procedures (Clontech). To obtain bacterial GST–EBNA5, bacterial cells (BL 21 Codon Plus strain; Stratagene) were transformed with pGEX and pGEX-EBNA5, respectively, and the expression of recombinant protein was induced with IPTG (final concentration of 0.5 mM). The cells were lysed in 20 mM Tris–HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.1% Tween, sonicated and centrifuged. DG75 cell extracts were prepared by lysing $2\times10^7$ cells in 500 ml 50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.1% Nonidet P-40 and centrifugation at 14000 $g$ for 10 min. All extracts were precleared by incubation with glutathione-conjugated Sepharose beads (Pharmacia) prior to the GST pull down experiments.

Fig. 1. Schematic diagram of plasmids encoding the bait protein (pAS2–EBNA5), yeast GST–EBNA5 expression vector (pYEX–EBNA5), bacterial GST–EBNA5 expression vector (pGEX–EBNA5) and the mammalian expression vector for a mini-EBNA5 molecule consisting of the W01, W2, Y1 and Y2 exons [pCI-E5(W01W2Y)]. The promoters of the expressed genes are indicated by arrows and the exons by open boxes.
the N-terminal 48 amino acid residues in HAX-1 do not participate in the interaction with EBNA5. SDS–PAGE and immunoblot analysis showed that yeast cells that carried a plasmid encoding the full-length HAX-1–AD fusion protein expressed a 50 kDa polypeptide recognized by both anti-HAX-1 and anti-GAL4-AD antibodies. The size of the protein corresponded well with the calculated molecular mass of a fusion between HAX-1 (35 kDa) and the GAL4 AD (15 kDa).

To confirm the interaction between HAX-1 and EBNA5, GST pull down experiments were performed. EBNA5 was expressed as a GST fusion protein in bacteria and in yeast and immobilized on glutathione-conjugated Sepharose beads. Beads carrying GST–EBNA5 and GST were incubated with extracts of DG75 cells, which constitutively express HAX-1, and sedimented by centrifugation, and the amounts of HAX-1 eluted from the beads and in the supernatant were determined by SDS–PAGE and immunoblot analysis using anti-HAX-1 antibodies. The results showed that GST–EBNA5 expressed in bacteria bound a significant fraction of the HAX-1 protein present in the DG75 cell extract (Fig. 2a, lane 5). GST-carrying beads did not bind HAX-1 (Fig. 2a, lane 3). Similar results were obtained with GST and GST–EBNA5 expressed in yeast cells (Fig. 2b). This indicates that the formation of a complex between EBNA5 and HAX-1 was not an effect of a yeast or bacterial cell-specific protein. Thus, on the basis of the two-hybrid analysis in yeast and the substantial fraction of cellular HAX-1 retained by the immobilized GST–EBNA5 fusion protein, the interaction between EBNA5 and HAX-1 clearly appears to be specific. A similar interaction between EBNA5 and HAX-1 in COS-7 cells was recently described by Kawaguchi et al. (2000).

HAX-1 has been found in the mitochondria, in the endoplasmatic reticulum and in the nuclear membrane (Gallagher et al., 2000; Suzuki et al., 1997). In B-cells, HAX-1 interacts with HS1, which is a component of the B-cell receptor (BCR) signalling pathway (Suzuki et al., 1997). In HeLa cells, HAX-1 interacts with cortactin, which is structurally related to HS1 and also localized in the cytoplasm (Gallagher et al., 2000). As the name implies, EBNA5 is generally regarded as a strictly nuclear protein. We have, however, found that the smallest species of the EBNA5 protein family, comprising a single W1W2 repeat domain and the unique Y1Y2 domain, is distributed in a patchy pattern exclusively in the cytoplasm of transfected DG75 cells (M. Dufva, A. Nerstedt & L. Rymo, unpublished experiments). Similar results were recently reported by Peng et al. (2000) and Kawaguchi et al. (2000). To determine the cellular localization of the E5(W0W2Y) protein and if possible substantiate the notion that the patchy staining pattern obtained with mini-EBNA5 corresponded to the distribution of mitochondria in the cytoplasm, DG75 cells transiently transfected with pCI-E5(W0W2Y) were stained in parallel with the EBNA5 monoclonal antibody JF186 and an antibody towards the mitochondrial matrix-associated protein hsp60. This protein is a useful marker for mitochondria since 80–85% of the total cellular amount of hsp60 is located in the mitochondria, while the remaining 20–15% is located in the endoplasmatic reticulum, the cell membrane and other sites (Soltys & Gupta, 1996). Our results showed that E5(W0W2Y) had a very similar cellular distribution to hsp60, indicating that E5(W0W2Y) is localized in the mitochondria (Fig. 3a–c). The antibody staining reactions appeared to be specific since pCI vector-transfected DG75 cells were only stained by the anti-hsp60 antibodies, and omission of either of the primary antibodies in the staining procedure resulted in a loss of the respective signal (data not shown). Staining DG75 cells for hsp60 and HAX-1 showed that HAX-1 also co-localized with hsp60 (Fig. 3d–f). In addition, the HAX-1 staining was distributed as a ring around the nucleus with no spatial relation to the hsp60 staining, suggesting that HAX-1 is also associated with the nuclear membrane. This is in accordance with a previous report addressing the localization of HAX-1 (Suzuki et al., 1997). The mitochondria were visualized by transfection of DG75 cells with a plasmid expressing cyan shifted mutant green fluorescent protein tagged with the mitochondrial localization signal from subunit VIII of cytochrome c oxidase.

Fig. 2. GST–EBNA5 fusion protein and HAX-1 interact in vitro. GST pull down experiments were performed as described (Ausubel et al., 1996, 1997). DG75 cell extract was incubated with GST– or GST–EBNA5-conjugated Sepharose beads and the relative amounts of HAX-1 in the bead fractions (denoted with B) and in the supernatants (denoted with S) were determined by SDS–PAGE and immunoblot analysis using mouse anti-HAX-1 antibody. Aliquots corresponding to 6 × 105 cells in the B lanes. Extract that had not been incubated with conjugated Sepharose beads was used as a positive control in lane 1. (a) GST and GST–EBNA5 proteins expressed in E. coli. (b) GST and GST–EBNA5 proteins expressed in yeast.
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Fig. 3. Co-localization of mini-EBNA5 and HAX-1 with hsp60 in transfected DG75 cells. DG75 cells (5 x 10⁶) were transfected with 2700 fmol of either pCI-E5(W₀₁W₂Y) or pCI control DNA. Hsp60 was detected in the cells by immunostaining using the goat anti-hsp60 antibody as the primary antibody and rhodamine-conjugated donkey anti-goat IgG antibody as the secondary antibody. The E5(W₀₁W₂Y) and HAX-1 proteins were detected using JF186 anti-EBNA5 and anti-HAX-1 antibodies, respectively, as primary antibodies, and FITC-conjugated rabbit anti-mouse IgG antibodies in a first step and FITC-conjugated swine anti-rabbit IgG antibodies in a second step as secondary antibodies. In (a)-(c), the nucleus was visualized with DAPI staining.

Fig. 4. Association between EBNA5 with one and seven W repeat domains, respectively, and HAX-1 in B-lymphoid cells. Extracts of DG75 cells transfected with the HAX-1 expression vector pME18sf-HAX-1, and the pCI, pCI-EBNA5 and pCI-E5(W₀₁W₂Y) plasmids as indicated (µg DNA per transfection) were immunoprecipitated using the JF186 anti-EBNA5 antibody and protein G–Sepharose beads. Proteins in the cell extract (T fraction), in the supernatant (S fraction) and eluted from the beads (B fraction) were analysed by SDS–PAGE and immunoblotting using a mouse anti-HAX-1 primary antibody and an alkaline phosphatase-conjugated secondary antibody. Aliquots corresponding to 6 x 10⁵ cells were applied in the S and T lanes and 30 x 10⁵ cells in the B lanes. The s lane contains the size markers, numbers are given in kDa.

The resulting staining pattern was very similar to that obtained for hsp60, HAX-1 and E5(W₀₁W₂Y) (data not shown).

To demonstrate a direct interaction between mini-EBNA5 and HAX-1 in the cells, we performed coimmunoprecipitation experiments. DG75 cells were cotransfected with the HAX-1 expression vector pMS18sf-HAX-1 and either the mini-EBNA5 expression vector pCI-E5(W₀₁W₂Y), the full-length EBNA5 expression vector pCI-EBNA5, or the empty pCI vector. EBNA5 proteins in the cell extract were immune precipitated with the JF186 anti-EBNA5 antibody, collected on protein G-conjugated Sepharose beads, and analysed by SDS–PAGE and immunoblotting with anti-HAX-1 antibody (Fig. 4). HAX-1 was consistently present in the immunoprecipitate obtained with EBNA5 with seven W₀₁W₂ domains as well as with mini-EBNA5. In fact, a larger fraction of the HAX-1 protein present in the extract was associated with the mini-EBNA5 than with the larger EBNA5 in five independent experiments. This observation does not necessarily mean that the mini-EBNA5 molecule has a higher affinity for HAX-1 than the larger EBNA5, but most likely reflects the fact that the former
molecule is located in the cytoplasm where it more readily could form a complex with HAX-1.

Published data indicate that most EBV-transformed LCLs and BL cell lines express only a few EBNA5 molecular species in the size range 42 to 78 kDa. This might indicate that smaller EBNA5 species are normally not expressed in EBV-infected cell lines. However, certain cell lines (B95-8 and BL41-E95) and newly infected tonsillar B-cells have been shown to express a 20 kDa EBNA5 protein (Finke et al., 1987). Transfection of DG75 cells with the pCI-E5(W\textsubscript{W\textsubscript{1}W\textsubscript{2}Y}) plasmid also resulted in the expression of a 20 kDa EBNA5 (data not shown). This suggests that the 20 kDa molecule in the pCI-E5(W\textsubscript{W\textsubscript{1}W\textsubscript{2}Y})-transfected cells and in newly infected cells contains only one copy of the W repeat domain together with the Y domain. The calculated molecular mass of an E5(W\textsubscript{W\textsubscript{1}W\textsubscript{2}Y}) molecule is only approximately 13 kDa, indicating that the mobility of the protein in SDS–PAGE does not correctly reflect its molecular mass. Thus, it seems probable that the small EBNA5 species studied in the present paper is identical to the small EBNA5 species present in large molar amounts early in EBV infection. The inability to detect the small EBNA5 molecules in most LCLs by immunological means might be explained by the fact that only one reactive epitope might be present on the molecule, which is the case when the JF186 anti-EBNA5 antibody is used. This is illustrated by our observation that more than 540 fmol of pCI-E5(W\textsubscript{W\textsubscript{1}W\textsubscript{2}Y}) had to be used in standard transfections in order to detect the E5(W\textsubscript{W\textsubscript{1}W\textsubscript{2}Y}) protein in transfected DG75 cells by immunostaining with the JF186 antibody. In contrast, transfection with 14 fmol of pCI-EBNA5 DNA, which is known to generate a short ladder of higher molecular mass EBNA5 molecules, resulted in easily detectable EBNA5 immunostaining. We know that there is a difference in expression at the transcriptional level between pCI-E5(W\textsubscript{W\textsubscript{1}W\textsubscript{2}Y}) and pCI-EBNA5 that results in the generation of four times more EBNA5 RNA per fmol of DNA on a molar basis with the latter vector. This explains part of the observed difference in the amounts of DNA needed for transfection with the two plasmids. However, a large part of the difference is presumably due to technical problems in the immunostaining method of the nature discussed above. Thus, it is quite possible that even if a significant number of small EBNA5 molecules are expressed in a cell line, they still might remain undetected. When analysing cells transfected with the pCI-E5(W\textsubscript{W\textsubscript{1}W\textsubscript{2}Y}) and pCI-EBNA5 expression vectors by immunostaining with the JF186 antibody, we have consistently found that, within the limitations of the sensitivity of the method, the 20 kDa EBNA5 molecules are exclusively located in the cytoplasm and the large EBNA5 molecules only in the nucleus. This is compatible with the idea that small and large EBNA5 molecules have distinct biochemical functions, possibly depending on the different locations but more likely due to properties inherent in the molecules themselves.

The functional significance of the interaction between EBNA5 and HAX-1 is difficult to evaluate since little is known about the function of HAX-1. The HAX-1 protein was first identified by a two-hybrid screen using the HS1 protein as bait (Suzuki et al., 1997). HS1 is a tyrosine kinase in the BCR signalling pathway and is important for the clonal expansion and deletion of B-cells (Fukuda et al., 1995; Taniuchi et al., 1995). Cross-linking of the BCR results in phosphorylation of HS1 by the BCR-associated tyrosine kinases Lyn and Syk (Yamanashi et al., 1997). The phosphorylation is necessary for targeting HS1 to the nucleus and to activate apoptosis (Yamanashi et al., 1997). One might speculate that E5(W\textsubscript{W\textsubscript{1}W\textsubscript{2}Y}), by interacting with HAX-1, might interfere with HS1 and BCR signalling to the nucleus that would otherwise induce apoptosis.

Homology searches revealed similarities between HAX-1 and the pro-apoptotic BNIP3 protein (Suzuki et al., 1997). In this context it might be worth noting that evidence has appeared supporting a role of EBNA5 also as a specific repressor of gene expression functioning by inhibiting the processing of pre-mRNA (M. Dufva, A. Nerstedt & L. Rymo, unpublished results). The effect of EBNA5 on the expression of cellular genes in a chromosomal context was analysed in transient transfections with an EBNA5 expression vector and using the DNA microarray technology. The experiments demonstrated that EBNA5 inhibited the expression of certain chromosomal genes, one of which coded for the BNIP3 protein. Interestingly, this protein interacts with anti-apoptotic proteins including BCL-2 and the EBV-encoded BCL-2 homologue BHRF1 (Boyd et al., 1994; Chen et al., 1997; Yasuda et al., 1998). BNIP3-binding of BCL-2 inhibits its anti-apoptotic function. Conceivably, down-regulation of BNIP3 by EBNA5 promotes the survival of EBV-infected cells.

We thank Dr T. Watanabe for the HAX-1 expression vector pME18sf-HAX-1 and Dr M. Masucci for the anti-EBNA5 antibody JF186. This investigation was supported by grants from the Swedish Medical Research Council (project 5667), the Swedish Cancer Society, and the Sahlgrenska University Hospital.

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Received 19 October 2000; Accepted 16 March 2001