Epstein–Barr virus encoded nuclear protein EBNA-3 binds XAP-2, a protein associated with Hepatitis B virus X antigen

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EBNA-3 (also called EBNA-3A) is one of the EBV encoded nuclear antigens that are necessary for B-cell transformation. EBNA-3 is known to target RBPs, nuclear proteins that also interacts with EBNA-2, EBNA-4 and EBNA-6. In order to identify additional EBNA-3 targets, an EBV-transformed human lymphocyte cDNA library was screened in the yeast two-hybrid system with N-terminus truncated EBNA-3 that cannot interact with RBP-J κ . A clone, encoding Xap-2 protein, a cellular partner of Hepatitis B virus X-antigen was isolated. This protein is also known as the p38 subunit of the aryl hydrocarbon receptor complex (ARA9). The specific binding to EBNA-3 was confirmed by showing that the GST-Xap-2 precipitated EBNA-3 from CV1 cells that were infected with recombinant vaccinia virus expressing EBNA-3. Deletion of the C-terminus of Xap-2 eliminated the binding. Fusion with green fluorescent protein showed that Xap-2 is preferentially cytoplasmic but translocates to the nucleus upon expression of EBNA-3. *Oncogene* (2000) 19, 1801–1806.

Keywords: EBNA-3; Xap-2; HBV

Introduction

EBV-immortalized lymphoblastoid cell lines (LCLs) express six nuclear antigens (EBNA 1–6), three latent membrane proteins (LMP 1, 2a, 2b) and two small RNAs (EBER 1–2). Concerted action of EBNA-1, -2, -3, -5 and -6 and LMP-1 is essential for immortalization and transformation of B-cells (Tomkinson *et al.*, 1993). EBNA-1 is transcriptional factor that is also required for the maintenance of the viral episome by binding to the latent origin of replication. EBNA-2 is strong transcriptional activator of several cellular and viral genes through the interaction with the cellular DNA binding protein RBP-J κ and RBP-2N. This interaction mimics constitutive Notch mediated signaling (Strobl *et al.*, 1997). The high molecular weight EBNA-3 (3, 4, 6 or as alternatively called 3a, 3b and 3c) are also able to bind RBP-J κ (Robertson *et al.*, 1996) and depending on the promoter context, modify the initiation of transcription (Allday *et al.*, 1993; Johannsen *et al.*, 1996; Waltzer *et al.*, 1996; Radkov *et al.*, 1997; Krauer *et al.*, 1998). EBNA-6 moreover has papilloma virus E7 like functions. EBNA-5 accumulates in the PML containing oncogenic domains

(PODs) where it co-localizes with the retinoblastoma tumor suppressor protein (Szekely *et al.*, 1996).

Identification of cellular proteins that can interact with the transformation associated EBNA-s is not yet complete. In the present study we have searched for cellular proteins that can bind to EBNA-3 in yeast two-hybrid system. Previously, RBP-J κ was identified as the major EBNA-3 interactive partner. It binds to the N-terminus of EBNA-3 (Krauer *et al.*, 1996, 1998; Robertson *et al.*, 1996; Zhao *et al.*, 1996). We have now used an N-terminus truncated EBNA-3 as bait that does not interact with RBP-J κ . Several interactive clones were identified. One was identical with the E-subunit of the TCP1 chaperonin complex (Kashuba *et al.*, 1999). Here we describe a clone that corresponds to the Xap-2 protein, a cellular target of the Hepatitis B virus X antigen, a transforming viral protein. Xap-2 is also known as a minor subunit of the aryl hydrocarbon receptor complex.

Results

Screening of human lymphocyte cDNA library with N-terminus truncated EBNA-3 identified an interactive clone (designated clone 436) that could grow on His, Leu and Trp deficient medium and expressed β -galactosidase from the protein–protein interaction dependent reporter locus. The strength of the binding was 14%, compared to the strongly interacting p53/LT positive control. Plasmid rescue from the yeast to *E. coli* resulted a clone that contained a 1053 bp long insert at its *XhoI* site.

Retransformation of clone 436 DNA together with the EBNA-3 construct into the SFY526 yeast strain confirmed the interaction. Plasmids containing EBNA-1, Δ EBNA-4, EBNA-5 and the empty pGBT9/BR vector were also retransformed together with the clone 436 DNA as negative controls. None of them showed any significant interaction, according to the β -galactosidase test (Table 1).

XhoI cleaved insert of clone 436 was used as a probe on Northern blots. It detected a transcript of approximately 1.5 kb. There was no significant difference in the expression levels in EBV transformed lymphoblastoid cell lines with or without mitomycin treatment in EBV negative or EBV positive Burkitt lymphoma lines with type I or type III viral latency (Figure 1).

Sequencing the insert showed that clone 436 was identical with a previously identified cellular gene that encodes Xap-2, a protein that associates with Hepatitis B virus X antigen (Kuzhandaivelu *et al.*, 1996).

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Table 1 Specific activity in β -galactosidase units of 436 co-transformants in yeast strain SFY 526

Target DNA in BD vector	β GU (abs. value)	% of large T-p53 activity*
EBNA-1	0.6	<1
EBNA-3	10.1	10
Δ EBNA-4	0.8	<1
EBNA-5	0.8	<1
Rb	0.6	<1
p53	0.6	<1
BD	0.8	<1

*Large T-p53 activity 72.8 (30% of standard deviation after four independent experiments)

Figure 1 Expression of Xap-2 in different lymphoid cell lines. Northern blot detected by the insert of clone 436 shows no difference in the expression levels in EBV transformed LCL (IARC171) with or without mitomycin (MMC) treatment; in EBV negative Burkitt lines (BL41 and BL28) their EBV positive convertants (BL41/95 and E95-B-BL28 respectively); in LMP-1 transfected BL41 (MTL11) and in the EBV positive Burkitt lines with type I or type III viral latency (MUTU cl99 and MUTU cl 148). Ethidium-bromide staining (bottom) shows the amount of RNA loaded

A human cDNA library (fetal brain) was screened with the 436 insert in order to isolate the full-length cDNA. Ten overlapping clones were isolated and assembled into a contig that included the 5' untranslated region, ATG and poly(A)⁺ tail. One of the cDNA clones covered the entire ORF (designed as Full436). It was identical with the frame of the yeast clone 436. The length of the longest cDNA clone was 1263 bp and encoded a 331 aa long protein. It contained an extra 150 bp long 5' untranslated region, compared to the published Xap-2 sequence.

Interestingly, the published aryl hydrocarbon receptor subunit (ARA9) (Carver *et al.*, 1997) 5' sequence and two EST sequences (HSC21B051 and HSC16G121) that are identical with Xap-2/clone 436 has an extended 5' sequence that differs from the Full436 5' untranslated region. To compare the expression pattern of messages with the two variant 5' regions, PCR primers were designed both from the Full436 and from the ARA9 5' region. They were combined with a common 3' primer located at

the beginning of the coding region. The amplified RT-PCR products were sequenced to confirm their identity. Full436 type transcripts were detected in brain, colon, kidney, lung and EBV immortalized lymphoblastoid cell lines (LCLs). ARA9 type transcripts were found in brain, colon and LCLs (Figure 2).

To investigate the protein-protein interaction *in vitro* the coding region corresponding to 16–331 amino acids of the ORF(1–331) was PCR amplified and inserted into glutathione-S-transferase bacterial expression vector (GST-2TK). A shorter GST construct containing 19–140 aa was also generated (Δ 436). Upon induction, 66 kDa and 45 kD fusion proteins were detected on silver or Coomassie blue stained SDS acrylamide gels.

These and several control GST fusion proteins were used to precipitate lysates of CV-1 cells infected with recombinant vaccinia virus containing full length EBNA-3 cDNA. Only GST-Full436, but not GST, GST-EBNA-5, GST-ARF or GST- Δ 436 precipitated EBNA-3 protein from the cell lysates (Figure 3). Recombinant EBNA-2 encoding vaccinia virus infected cell lysates were included as non-specific precipitation controls.

To study the subcellular localization of the protein Full436 was cloned into the green fluorescence protein fusion vector pEGFP-C1 and into pBabe-HA expression vector. The constructs were transfected into CV1, MCF7, NIH3T3, DG75 and BJAB cells. Protein expression was detectable already after 4–6 h by direct fluorescence (GFP) or immunostaining (HA epitop tag). The protein was predominantly localized to the cytoplasm in all cell lines. CV-1 and NIH3T3 cells transfected with GFP-Full436 were superinfected with recombinant vaccinia expressing EBNA-3 or EBNA-2. EBNA-3 but not EBNA-2 induced nuclear translocation of the GFP-Full436 fusion protein. In the nucleus the two proteins often showed speckled distribution with high degree co-localization (Figure 4).

Discussion

Aryl hydrocarbon receptor (AhR) is a phylogenetically ancient protein that is present in all living vertebrate groups, with a possible invertebrate homologue (Hahn *et al.*, 1997). AhR is a ligand-activated transcription factor, a member of helix-loop-helix/basic region transcription factor family. No physiological AhR ligands have been identified yet. Halogenated polycyclic aromatic hydrocarbons, originating from the environment, were shown to act as ligands (Yamaguchi *et al.*, 1997). AhR is 95 kD protein localized mainly to the cytoplasm. When bound to one of its exogenous ligands, TCDD (2,3,7,8-tetrachloro dibenzo dioxine), most of the AhR translocates to the nucleus where it forms a heterodimer with the Arnt (AhR nuclear translocator) protein that binds to DNA. Arnt probably associates with transcriptional co-activator CBP/p300. (Kobayashi *et al.*, 1997; Vaziri *et al.*, 1997).

In cells that are not exposed to aryl hydrocarbon ligands, AhR forms a complex with heat shock protein hsp90 and a minor subunit, p38 or ARA9.

Figure 2 (a) Schematic view of alternative 5' untranslated region of ARA9/Xap2 and Full436 mRNA with the specific 5' and common 3' primers. In RT-PCR these primers amplify various 5' fragments of Xap2/436 mRNA from different cDNA sources. The samples are the following: 1, 2 and 13 no CDNA (water control), 3 and 4 lung, 5 and 6 kidney, 7 and 8 colon carcinoma (SW480), 9 and 10 brain, 11 and 12 EBV transformed B cells (LCL970402). Samples of 1, 3, 5, 7, 9, 12 were amplified using the ARA9 specific 5' primer whereas 2, 4, 6, 8, 10, 11, 13 were amplified using the Full436 specific 5' primer. (b) Nucleotide and amino acid sequence of the Full436 clone. The variant 5' untranslated region that differs from the ARA9 transcript is underlined

Figure 3 GST-Full436 but not different control fusion proteins precipitates EBNA-3 from CV-1 cell lysates infected with recombinant vaccinia virus as detected by Western blotting

The three tetratricopeptide repeats found in the C-terminus of p38 are necessary and sufficient for interaction with the AHR complex (Carver *et al.*,

1998). The p38 subunit is identical with Xap-2, a cellular protein that also binds the N-terminus of Hepatitis B virus X antigen (Meyer *et al.*, 1998). Co-transfection of Xap-2 with X antigen inhibits the X mediated transcriptional transactivation in CAT assays (Kuzhandaivelu *et al.*, 1996).

In the present study we showed that p38/Xap-2 interacts specifically with EBNA-3 in the yeast two-hybrid system and in GST pull down assays. The predominantly cytoplasmic p38/Xap-2 translocates to the nucleus upon expression of EBNA-3. It will be important to explore whether the interaction of EBNA-3 with p38/Xap-2 alters the intracellular distribution of AhR.

The binding of p38/Xap-2 to the transforming proteins of two evolutionarily very distant viruses may indicate an involvement of the arylhydrocarbon receptor signal transduction pathway in virus induced cell transformation.

Figure 4 GFP-Full436 localizes to the cytoplasm of transfected NIH3T3 cells (a) Upon infection with EBNA-2 (red) expressing recombinant vaccinia virus (rVV) GFP-Full436 remains cytoplasmic (b) but accumulates in the nucleus upon infection with EBNA-3 (red) rVV where it colocalizes with EBNA-3 (c). DNA staining with 33258 is blue

Materials and methods

Plasmids

The polylinker region of GAL-4 DNA-binding domain containing vector pGBT9 and of GAL-4-activation domain containing vector pGAD10 (both from Clontech) were modified to be compatible with the GST-2TK expression vector (designated as BD and AD respectively).

PBabe-HA vector was created by inserting an oligonucleotide encoding the Cosak sequence, ATG and affinity tag epitope from the influenza virus hemagglutinin protein into the *EcoRI*–*Bam*HI site of the pBabe-puro retroviral expression vector.

Construction of the plasmids *DNEBNA-3/BD*, *EBNA-1/BD*, *ΔEBNA-4/BD*, *EBNA-5/BD*, *ΔEBNA-1/BD* (lacking Ala-Gly repeat), *Rb/BD*, *Rb/AD*, *p53/AD*, *EBNA-5/GST-*

2TK, Δ EBNA-1/GST-2TK was previously described (Kashuba *et al.*, 1999).

N-terminus truncated mouse p53 in pGBT9 (pVA3) and SV40 Large T-antigen in pGAD10 (pTD1, both from Clontech) were used as positive interaction controls.

N-terminus EBNA-3/BD was generated by inserting the 477 bp PCR product, corresponding to amino acids 3–159 of EBNA-3 cDNA.

C-terminus EBNA6/GST-2TK was generated by inserting a 590 bp fragment corresponding to amino acids 840–1036 of EBNA-6 cDNA. ARF-GST-2TK plasmids were generous gifts of Klas G Wiman.

The Full436/GST-2TK, Full436/pEGFP-C1, Full436/pBabe-HA were generated by inserting the 1029 bp PCR-product into the respective vectors (5' primer GCCAGATCTCGTGTGATACAGGAAGGC; 3' primer ACGAATTCACAGAAGCATGACGCA), corresponding to amino acids 16–331 of the ORF of the 436 clone.

Δ 436/GST2TK, Δ 436/pEGFP-C1 were generated by inserting the 371 bp PCR-product (5' primer ATGGATCC-CAGGAAGGCCGAGGA; 3' primer CAGAATTCGGCGTCCAGGTCAGCATG), corresponding to amino acids 20–140 of ORF for 436 clone.

Yeast strains and cDNA library screening

The *Saccharomyces cerevisiae* HF7c strain was used for library screening and SFY526 for conformation of the interaction upon retransformation. Human lymphocyte MATCHMAKER cDNA library in pACT GAL-4 transcriptional activation domain vector along with the yeast strains were obtained from Clontech. Library screening was run according to the Clontech protocol. Interacting clones were selected on SD plates lacking His, Leu and Trp. The fastest growing clones were further tested for β -galactosidase activity by ONPG test as described (Kaise *et al.*, 1994). Specific activity of the given clones was calculated as percentage of β -galactosidase units of the positive control. The samples were incubated with ONPG at 30°C for 2 h.

Sequencing

Sequencing was done using automated ALF sequencing machine (Pharmacia) or capillary Apply BioSystem sequence machine (Perkin-Elmer).

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PCR

PCR was carried using Perkin-Elmer or Idahotech thermocyclers. Primers to amplify the variant 5' sequences: Full436 specific 5' primer: CACCATTGAGAGGAACC; ARA9/Xap2 specific 5' primer: AAGCTACCGAGGTGCTA; Common 3' primer: CTTGCCCAACCGCGAT.

Cells and cell culture

All cell lines were cultured at 37°C, in Iscove's medium containing 10% fetal calf serum and 50 mg/ml Gentamycin. Periodic staining with Hoechst 33258 monitored the absence of mycoplasma. CV1 cells were infected at high multiplicity with recombinant vaccinia virus encoding EBNA-3 or EBNA-2 as described (Kashuba *et al.*, 1999). CV1, NIH3T3, and EBNA-3 or EBNA-4 expressing BJAB cells were transfected with NGFP-Full436 construct using polyethylenimine (PEI) or electroporation (230 V, 960 μ F, 38 ms in volume of 300 μ l 10% FCS containing medium in the presence of 1.25% DMSO) respectively. Infection with recombinant vaccinia viruses was done as described (Gavioli *et al.*, 1992).

GST pull down assay

GST pull down assay was performed as described (Kashuba *et al.*, 1999). All cell lysate contained additional 0.5% of BSA as a specific competitor.

Immunofluorescence staining

Immunostaining and digital image capturing was carried out as described (Szekely *et al.*, 1996, 1997, 1998) using the anti-EBNA-3A monoclonal antibody T2.78-19.

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