Regulation of Transactivation Function of the Aryl Hydrocarbon Receptor by the Epstein-Barr Virus-encoded EBNA-3 Protein*

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EBNA-3 is one of the Epstein-Barr virus (EBV)-encoded nuclear antigens that is indispensable for immunoblastic transformation and sustained proliferation of B-lymphocytes. The molecular mechanisms responsible for the function of EBNA-3 are poorly understood. We previously found that EBNA-3 interacts with an immunophilin-like protein XAP2/ARA9/AIP, which in mammalian cells is known to interact with the latent aryl hydrocarbon receptor (AhR). AhR is a ligand-inducible transcription factor that mediates cellular responses to environmental pollutants, such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). In this study, we show that EBNA-3 interacts specifically with AhR. The stability of this interaction is determined by the activation state of AhR and its association with XAP2. We and others have demonstrated that XAP2 retains the nonactivated AhR in the cell cytoplasm. However, in the presence of TCDD, the effect of XAP2 on the intracellular localization of AhR was counter-acted by EBNA-3, resulting in nuclear translocation of the AhR. In addition, EBNA-3 enhanced transactivation function by the ligand-activated AhR in cells, as assessed by reporter gene assays. Our data suggested that EBNA-3 plays a role in facilitating the ligand-dependent AhR activation process. Following activation of the AhR, we also observed that EBNA-3 counteracted the inhibitory effect of TCDD on the growth of EBV-carrying lymphoblasts. Taken together, our studies revealed a novel interaction between EBV- and AhR-dependent cellular pathways that control cell proliferation and survival.

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Epstein-Barr virus (EBV) is a γ-herpesvirus that infects more than 90% of the human population. It is the causative agent of infectious mononucleosis and of lymphoproliferative disease in immunosuppressed hosts. In vitro infected B-cells are transformed into continuously proliferating lymphoblastoid cell lines (LCLs) that express nine EBV-encoded proteins as follows: six nuclear antigens (EBNA1–6), three membrane proteins (LMP1, LMP2A, and -2B), and two small nonpolyadenylated RNAs (EBER1–2). The same pattern of protein expression is found in the B-blasts that proliferate in infectious mononucleosis and post-transplant lymphoproliferative disease. Six of the nine proteins are required for the efficient transformation of B-lymphocytes by EBV: EBNA-1, EBNA-2, EBNA-3 (EBNA-3A), EBNA-5 (EBNA-LP), EBNA-6 (EBNA-3C), and LMP1 (for review see Refs. 1 and 2).

EBNA-3 is not only indispensable for B-cell transformation but is also implicated in the maintenance of LCL proliferation. It was recently shown that 3–5-fold overexpression of EBNA-3 leads to the down-regulation of c-myc, CD21, and CD23 and induces G0/G1 arrest in LCLs (3). In another study, LCLs were established by infecting primary B-lymphocytes with the recombinant EBV that expresses EBNA-3 fused to the hormone-binding domain of a 4-hydroxysteroidoxifen-dependent mutant estrogen receptor (EBNA-3AHT). Withdrawal of 4-hydroxysteroidoxifen from cell culture medium results in cell death, despite the fact that the expression levels of c-myc remain high (4).

The molecular mechanisms of EBNA-3 action are poorly understood. It is reportedly involved in transcriptional regulation, interacting with RBP-Jκ (5–7) and CtBP (8). We have shown previously that EBNA-3 may interfere with a growth-associated metabolic pathway through its binding to a uridine kinase/uracil phosphoribosyltransferase, UK/UPRT (9). On the base of its binding to the immunophilin-like XAP2/ARA9/AIP protein, a minor subunit of the nonactivated (latent) aryl hydrocarbon-dioxin receptor (AhR/DR) complex (10–13), we have also suggested that EBNA-3 may be involved in the regulation of xenobiotic signal transduction pathways.

AhR is a ligand-inducible transcription factor that mediates cellular response to xenobiotic compounds such as environmental pollutants, e.g. polycyclic aromatic hydrocarbons and polychlorinated dioxins, most notably 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). The receptor belongs to the basic helix-loop-helix (bHLH)/Per-ARNT-Sim domain (PAS) family of transcription factors (for review see Ref. 14). Two conserved domains characterize these proteins, the N-terminal bHLH DNA binding domain and the PAS domain, which spans two hydrophobic repeats termed PAS-A and PAS-B. AhR is unique among bHLH/PAS proteins, because it contains a ligand-binding region located in the C-terminal part of the PAS domain, including the PAS-B motif. In the absence of ligand, the latent receptor is associated with the molecular chaperone Hsp90 and two Hsp90-binding proteins, the co-chaperone p23 and XAP2 (11–13, 15). Hsp90 maintains a high affinity
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ligand-binding conformation of the receptor, whereas p23 is thought to stabilize the latent receptor-Hsp90 heterocomplex. XAP2, in turn, stabilizes the AhR protein (16) and participates in the regulation of the intracellular localization of the receptor by an uncharacterized cytoplasmic retention mechanism (15, 16). Upon ligand binding, AhR accumulates in the nucleus where it forms a transcriptionally active complex with the bHLH/PAS transcription factor ARNT. Dimerization with ARNT induces the release of the Hsp90 complex from the receptor (17). The AhR-ARNT heterodimer activates the transcription of target genes by specific binding to xenobiotic-inducible transcriptional control elements, XREs. They are located in the regulatory regions of a gene network encoding drug-metabolizing enzymes such as cytochrome P450Ia1 (for review, see Ref. 14). Activation of the AhR-mediated signaling pathway by xenobiotic compounds in animal model systems (mainly in rodents) has numerous toxic consequences, including carcinogenesis, immunosuppression, thymic involution, severe wasting, and death (for review, see Ref. 18). Transgenic mice expressing a constitutively active mutant AhR develop tumors in the glandular part of the stomach (19), consistent with an oncogenic function of the activated receptor.

Given the common interaction with XAP2, it is possible EBNA-3 may modulate the AhR signal transduction pathway. Here we demonstrate that EBNA-3 specifically interacts with the AhR protein. The stability of this interaction depended on the activation state of the receptor and on the presence of XAP2. EBNA-3 expression increases the activation of transcription by AhR in reporter gene assays. Because both AhR and EBNA-3 are involved in the regulation of cell proliferation, we have also examined the effects of TCDD on the growth of EBV-transformed and control B-lymphocytes in the presence and absence of EBNA-3, and we found that EBNA-3 counteracted the inhibitory effect of TCDD on the growth of virally transformed immunoblasts.

MATERIALS AND METHODS

Recombinant Plasmids—pBC vectors (encoding the GST tag), pBC/DR construct (encoding GST-AhR), pSG5/XAP2 (15), pCMX/DR-GFP (encoding AhR-GFP), pCMV2/FLAG (encoding the FLAG peptide (Eastman Kodak Co.), pCMV2/FLAG-XAP2 (16), pGFP-XAP2 (10), pGEM7/ARNT (20), and pTXIXI (21) have been described previously. pBabe-EBNA-3, encoding the N-terminal deletion mutant of EBNA-3 (Δ1–127), was constructed by inserting a BamHI-Xhol fragment of EBNA-3 cDNA from pZIP-EBNA-3 (kind gift of E. Kieff) into the BamHI-EcoRI-digested pBabe-puro vector by using an EcoRI/Xhol adaptor. pBabe-EBNA-3 encodes residues 128–912 of the EBNA-3 protein. To generate pCMV2/FLAG-EBNA-3, first the 2463-bp PCR fragment was created using pBabe-EBNA-3 as a template. Subsequently, the PCR product was digested by HindIII-Xbal and ligated in-frame with the FLAG epitope into pCMV2-FLAG vector digested with HindIII-Xbal.

Cell Lines—Cell lines used in this study were as follows: MCF7 (human breast adenocarcinoma cells), COS7 (African green monkey kidney fibroblasts, transformants of CV-1 cells by an origin-defective mutant of SV40 virus, expressing SV40 large T antigen), DG75, EBV-negative Burkitt’s lymphoma, DG75 sub-lines that express EBNA-3 or EBNA-5 constitutively (both transfectants were the kind gifts of Lars Rymo); LCL cells were freshly established from tonsil B-cells as described previously (22). Lymphoid cell lines were grown in Iscove’s culture medium, supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 μg/ml penicillin, and 100 μg/ml streptomycin. COS7 and MCF7 cells were grown in Dulbecco’s minimum essential medium (DMEM), supplemented with 10% fetal bovine serum, 100 μg/ml penicillin, and 100 μg/ml streptomycin.

Protein Expression In Vitro—In vitro translation of GST-AhR and [35S]methionine-labeled EBNA-3 was carried out using coupled transcription/translation reactions in rabbit reticulocyte lysate (Promega) according to the manufacturer’s recommendations.

Immunostainings—Cells, grown on 2 × 2-cm glass coverslips, were fixed by submerging them into a mixture of methanol and acetone (1:1) at −20 °C for at least 1 h. Immunostaining was carried out as described previously (23). Primary antibodies, used for immunostaining, were as follows: mouse monoclonal antibody against FLAG epitope (Sigma), mouse antibodies against EBNA-3 T278-19 (kind gift of M. Rowe), rabbit serum against XAP2 (described below), and rabbit serum against EBNA-3 (ASLA Ltd.). Conjugated secondary antibodies were as follows: Texas Red-conjugated horse anti-mouse Ig (Vector Laboratories), TRITC-conjugated swine anti-rabbit serum (DAKO), fluorescein isothiocyanate-conjugated swine anti-rabbit serum (DAKO), AMKA-streptavidin (Vector Laboratories), and biotinylated goat anti-rabbit serum (DAKO). For DNA staining, 0.4 μg/ml bisbenzimide (Hoechst 33258 from Sigma) was added together with the secondary antibody.

Microscopy, Photo and Image Analyses—For routine documentation, the images were captured using a DAS microscope Leitz DM RB with a Hamamatsu dual mode cooled charged coupled device camera C4880 as described elsewhere (23).

Cell Extracts and Immunoblotting—For in vivo immunoprecipitation experiments, COS7 cells were grown in 10-cm diameter dishes. Expression vectors encoding recombinant proteins were transiently transfected by using Lipofectamine (Invitrogen) or FuGENE 6 (Roche Applied Science), according to the manufacturers’ protocols. Infection with recombinant vaccinia virus was carried out for 24 h as described (24). To prepare whole cell extracts (WCEs), cells were washed twice with cold phosphate-buffered saline (PBS), collected by centrifugation, and resuspended in lysis buffer (50 mM Tris-HCl, pH 7.4, 50 mM NaCl, 10% glycerol, and 0.5% Tween 20) supplemented with a protease inhibitor mixture (‘Complete-Mini’, Roche Applied Science). Cell suspensions were sonicated by two 4-s bursts. Lysates were cleared by centrifugation for 30 min at 13,000 × g at 4 °C. WCE protein (600 – 800 μg) was incubated at 4 °C for 12–18 h with either anti-FLAG or anti-GST (Amersham Biosciences) antibodies, as indicated in the figure legends. Immune complexes were precipitated by adding 25 μl of a 50% slurry of protein A-Sepharose (Amersham Biosciences), followed by incubation at 4 °C under slow rotation for 90 min. After rapid centrifugation, the resulting pellets were washed four times with 1 ml of cold lysis buffer. Immunoprecipitated proteins and whole cell extracts were analyzed by 7.5 or 12% SDS-PAGE and transferred to nitrocellulose membranes. Immobilized proteins were incubated for 2 h at 25 °C with primary rabbit polyclonal anti-XAP2 (dilution 1:2000), mouse monoclonal anti-FLAG (1:2000), mouse monoclonal anti-EBNA-3 (1:500), mouse monoclonal anti-Hsp90 (25), or rabbit polyclonal anti-AhR antibodies (Biomol, 1:500) in blocking solution (5% nonfat milk in phosphate-buffered saline). Horseradish peroxidase-conjugated anti-rabbit (Dako) or anti-mouse (Amersham Biosciences) immunoglobulins were used as secondary antibodies diluted 1:1000 in blocking solution. After extensive washing in phosphate-buffered saline containing 0.2% Tween 20, immunocomplexes were visualized using enhanced chemiluminescence reagents (Amersham Biosciences).

Reporter Assays—MCF7 cells were seeded into 6-well plates at about 60% confluency before transfection. Cells were transfected with 0.5 μg of the XRE-thymidine kinase-luciferase reporter plasmid pTXIXI and...
different amounts (from 100 to 1250 ng) of the EBNA-3 encoding pCMV2/FLAG-EBNA-3 construct or empty pCMV2/FLAG vector in control cells by using Lipofectamine (Invitrogen) and serum-free DMEM according to the manufacturer’s recommendations. After 6 h of transfection, the medium was replaced with 10% fetal calf serum containing DMEM and grown in the presence of 10 nM 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD; Chemsyn, Lenexa, Kans.) or vehicle (dimethyl sulfoxide, Me2SO) alone for 24 h. Cells were collected in PBS by scraping, and extracts were analyzed in the presence of ATP and luciferin reagents for luciferase activity by using a luminometer.

Production of Rabbit Anti-XAP2 Serum—A 42-mer oligopeptide corresponding to residues 289–330 of the human XAP2 protein sequence was synthesized by ThermoHybaid (Thermo Biosciences GmbH, Ulm, Germany). Chinchilla female rabbits (2.5–2.8 kg, 8–10 weeks old) were primed by two subcutaneous injections of 100 μg of the XAP2 peptide dissolved in 400 μl of PBS mixed with complete Freund’s adjuvant (Sigma) (1:1 v/v) at weeks 0 and 1. Boosts were done intravenously with 50 μg of XAP2 peptide in 200 μl of PBS mixed with incomplete Freund’s adjuvant (1:1, v/v) at weeks 4 and 8. Blood was collected 12 days after the second boost. Serum reactivity and specificity was tested by indirect ELISA on 96-well plates (MaxiSorp, Nunc, Denmark) coated with XAP2 peptide or unrelated antigens using a protocol described previously (26).

RESULTS

EBNA-3 Interacts with AhR In Vitro and in Vivo—In initial experiments, we examined whether EBNA-3 and AhR proteins could physically interact with each other. We used a rabbit reticulocyte lysate-based in vitro transcription/translation system to produce GST-fused AhR and [35S]methionine-labeled EBNA-3. A mixture of both proteins was incubated at room temperature for 1 h in parallel with a control reaction mixture containing EBNA-3 and mock-translated material (Fig. 1A). Proteins interacting with GST-AhR were subsequently co-immunoprecipitated using anti-GST antibodies (α-GST lpp). Electrophoretically separated immune complexes were analyzed by fluorography to detect the GST-AhR-bound EBNA-3. C, GST-AhR or GST tag alone were transiently expressed in COS7 cells, which were infected with recombinant vaccinia-EBNA-3 virus. Expression levels of GST-AhR and EBNA-3 were analyzed by immunoblotting aliquots (25 μg of protein) of WCEs using anti-AhR (upper panel) and anti-EBNA-3 antibodies (lower panel), respectively. D, EBNA-3 was co-immunoprecipitated together with GST-AhR using anti-GST antibodies. Electrophoretically separated immune complexes were analyzed by immunoblotting using anti-AhR and anti-EBNA-3 antibodies to detect AhR (upper panel) and EBNA-3 (lower panel), respectively. The positions of molecular weight markers, GST-tagged AhR (GST-AhR), and EBNA-3 (EBNA-3) are indicated.
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Figure 3. Intracellular localization of co-expressed AhR, EBNA-3, and XAP2 (double transfecants). AhR-GFP (1 μg), EBNA-3 (1 μg), GFP-XAP2 (0.5 μg), and FLAG-XAP2 (0.5 μg) were expressed in pairs in MCF7 cells and subsequently incubated with TCDD or vehicle (Me2SO) alone for 20 h. Cells were then fixed and incubated with appropriate antibodies as indicated under “Materials and Methods.” For detection of EBNA-3, mouse monoclonal anti-EBNA-3 antibody was used as a primary antibody, whereas the Texas Red-conjugated anti-mouse IgG antibody was applied as a secondary antibody. For detection of FLAG-XAP2, rabbit anti-XAP2 antibodies were used as primary antibodies, whereas anti-rabbit TRITC-conjugated anti-rabbit IgG antibodies were applied as secondary antibody. In order to visualize cell nuclei/DNA, cells were stained with the Hoechst reagent. The 3rd and 4th row of panels show AhR-GFP (green, panels m and n) co-expression with EBNA-3 (red, panels s and t), AhR-GFP (green, panels o and p) co-expression with XAP2 (red, panels u and v), and GFP-XAP2 (green, panels q and r) co-expression with EBNA-3 (red, panels w and x). EBNA-3, Hoechst staining is demonstrated in panels g–i. The merged picture of Hoechst staining with green-stained protein is shown in the 1st row of panels (a–f). The merged picture of the co-expressed proteins is shown in the bottom row of panels (y, z, and z1–4).

EBNA-3 was specifically recovered in a complex formed by GST-AhR (lanes 1 and 2). The interaction between EBNA-3 and AhR proteins was also investigated in living cells. EBNA-3 was overexpressed in COS7 cells using recombinant vaccinia virus, whereas the GST-AhR fusion protein, as well as its co-chaperone XAP2, were transiently introduced into cells by lipofection (Fig. 1C). GST-AhR was precipitated from WCEs with anti-GST antibodies. Consistent with the above results, EBNA-3 specifically interacted with GST-tagged AhR (Fig. 1D, lanes 1 and 2). Protein levels of GST-AhR were higher in the presence of XAP2, which is known to stabilize protein levels of the receptor (Fig. 1C, compare lanes 2 and 4). It is also noteworthy that the recovery of EBNA-3 from the XAP2-containing lysate was less efficient compared with control reactions lacking overexpressed XAP2 (Fig. 1D, compare lanes 2 and 4). These results show that EBNA-3 interacted specifically with the recombinant AhR protein and that the stability of this interaction can be influenced by XAP2, a common binding protein for both AhR and EBNA-3.

AhR, EBNA-3, and XAP2 Affect the Intracellular Localization of Each Other—It was recently shown that XAP2 regulates the intracellular localization of AhR by distributing it throughout the cytoplasmic compartment of the cell (15, 16). When co-expressed with EBNA-3, the localization of XAP2 changed from diffuse and exclusively cytoplasmic to spot-like nuclear conglomerates with extensive co-localization with EBNA-3 (10). To investigate whether the intracellular distribution pattern of the AhR and the AhR-XAP2 complex were influenced by the presence of EBNA-3, we co-expressed recombinant AhR-GFP fusion protein and FLAG tagged and nontagged EBNA-3 and XAP2 proteins in various combinations in MCF7 cells. Intracellular localization of these proteins was determined by combining immunostaining with fluorescent microscopy as indicated in legends of Figs. 2, 3, and 4 (see also “Materials and Methods” for experimental details).

In single protein expression studies (Fig. 2), GFP-tagged AhR was evenly distributed in both the cell cytoplasm and the nucleus (panel g); EBNA-3 displayed spot-like nuclear conglomerates (panel i) similar to those observed earlier in COS1 cells (10), whereas XAP2 was localized mainly in the cell cytoplasm (panel k). Upon exposure to TCDD, AhR was redistributed into the cell nucleus (Fig. 2, panel h), but no detectable changes were observed in the subcellular distribution of EBNA-3 and XAP2 (panels j and l, respectively). In cells transiently expressing AhR-GFP together with EBNA-3, the intracellular localization of the AhR-GFP construct was similar to what was observed in the absence of overexpressed EBNA-3 (Fig. 2, panel g, and Fig. 3, panel m). At the same time, EBNA-3 was more diffusely distributed in the presence of AhR (Fig. 3, panels s and t) compared with EBNA-3 expression alone (Fig. 2, panels i and j). In line with earlier published observations (15, 16), co-expression of XAP2 led to redistribution of the nonactivated AhR-GFP to the cell cytoplasm as well as a very low nuclear accumulation rate of the receptor in the presence of TCDD (Fig. 3, panels o and p). In the case of XAP2 co-expression with EBNA-3, part of XAP2 remained in the cell cytoplasm, but it also formed spot-like nuclear conglomerates wherever it co-localized with EBNA-3 (Fig. 3, panels q and r; a similar result has also been observed in COS51 cells (10)). This suggests that XAP2 follows the nuclear localization pattern of EBNA-3, once it has entered the nucleus.

Fig. 4 shows the co-expression of the following three examined proteins: AhR-GFP, EBNA-3, and XAP2. Similarly to what was observed in Fig. 3, the intracellular localization of nonactivated AhR-GFP was determined by XAP2, regardless of the presence of EBNA-3; most of the AhR-GFP fusion construct was localized in the cell cytoplasm (Fig. 4, panels e and f). However, upon treatment of the cells with TCDD, AhR-GFP efficiently migrated to the cell nucleus (Fig. 4, panels g and h), although the process of AhR-GFP nuclear translocation was somewhat less effective, compared with cells that expressed only AhR (see panel h in Fig. 2). This indicates that during the process of ligand-dependent activation of AhR, the effect of cytoplasmic retention of AhR by XAP2 is overcome by EBNA-3, perhaps involving direct interaction of EBNA-3 with the ligand-bound receptor. Notably, the spot-like nuclear conglomerates of XAP2, observed in EBNA-3 and XAP2 co-expression experiments in Fig. 3 (panels q and r), were not detected when AhR-GFP was co-expressed with the two proteins (panels i–l).
XAP2 (panels q–t), suggesting that the complex generated by the two EBNA-3-interacting proteins, AhR and XAP2, was able to recruit EBNA-3 to the cytoplasm. Moreover, in the presence of both AhR and XAP2, EBNA-3 lost its characteristic pattern as spot-like nuclear conglomerates and also, as already noted above, the ability to induce the accumulation of XAP2 conglomerates that were observed in the co-expression of EBNA-3 and XAP2 in the absence of AhR (Fig. 3, panels w and q, respectively).

The Stability of the Interaction between EBNA-3 and AhR Is Determined by the Activation State of AhR and the Presence of the Co-chaperone XAP2—The present results indicate that, depending on the activation state of AhR, EBNA-3 and XAP2 dominate each other in regulating the intracellular localization of AhR. We therefore performed a series of protein-protein interaction assays in order to find out whether XAP2 and EBNA-3 competed for AhR binding and whether AhR-EBNA-3 and AhR-XAP2 complexes were affected by AhR ligand treatment. First, we wanted to examine the effects of EBNA-3 on complex formation between AhR and XAP2. The AhR-GFP fusion protein was expressed in MCF7 cells in the presence or absence of FLAG-tagged XAP2 and in the presence or absence of different amounts of EBNA-3 (Fig. 5A). Anti-FLAG antibody-mediated co-immunoprecipitation of AhR-GFP FLAG-XAP2 complexes from WCEs showed that AhR binding to XAP2 was unaffected by EBNA-3 (Fig. 5B). In the reverse experiment, AhR-GFP was co-expressed together with constant levels of FLAG-tagged EBNA-3 and different amounts of XAP2 (Fig. 5C). In this case, we observed a disruption of the EBNA-3-AhR-GFP complex by XAP2 in a concentration-dependent manner (Fig. 5D).

In order to test the effect of TCDD on AhR binding to EBNA-3, we expressed wild type AhR and FLAG-tagged EBNA-3 in COS7 cells in the presence or absence of ARNT and/or XAP2-GFP (Fig. 6A). Following exposure to TCDD or to vehicle (Me3SO) alone, WCEs were prepared, and FLAG-EBNA-3-associated AhR was co-immunoprecipitated by anti-FLAG antibodies (Fig. 6B). Our results indicate that AhR interacted with EBNA-3 with equal efficiency in the presence and absence of ligand (Fig. 6B, lanes 3 and 4). Most interestingly, in the presence of the overexpressed ARNT, EBNA-3 interaction with ligand-activated AhR was more stable than with the latent (nonactivated) form of the receptor (Fig. 6B, lanes 5 and 6). In excellent agreement with the

![Figure 4: Co-expression of AhR, EBNA-3, and XAP2 (triple transfectants).](Image)

![Figure 5: XAP2 inhibits EBNA-3 binding to AhR.](Image)
observations in Fig. 5, C and D, the AhR-EBNA-3 complex was destabilized in the presence of XAP2. However, this was the case only when AhR was not activated by ligand (Fig. 6B, lanes 7 and 9) because no effect of XAP2 was observed in the presence of TCDD (Fig. 6B, lanes 8 and 10). Taken together, these results demonstrate that XAP2 prevents EBNA-3 from binding to the nonactivated AhR complex, despite its ability to interact with EBNA-3 directly. This XAP2-mediated blockade, however, is removed upon activation of the receptor by ligand.

The Interaction of EBNA-3 with Hsp90 Is Mediated via Association with XAP2—Stable interaction between XAP2 and latent AhR complex is mediated through the binding to the molecular chaperone Hsp90 (27). A possible interaction between EBNA-3 and Hsp90 has not been investigated so far. Therefore, a protein-protein interaction experiment was carried out to address this issue. FLAG-tagged EBNA-3 was expressed in COS7 cells (Fig. 7A, middle panel, lanes 2 and 3 and 7–10) in the presence or absence of AhR (upper panel) and in the presence or absence of XAP2 (lower panel). We also used FLAG-tagged XAP2 (Fig. 7A, lower panel, lanes 4, 11, and 12), which served as a positive control for the detection of target protein binding to the chaperone. Following treatment of cells with or without TCDD (as indicated in Fig. 7A), FLAG-EBNA-3 and FLAG-XAP2 proteins were immunoprecipitated from WCEs using anti-FLAG antibodies. Immune complexes were subsequently analyzed by immunoblotting by using antibodies against Hsp90 in the absence or presence of AhR (lanes 1 and 5–8). In contrast, FLAG-XAP2 efficiently interacted with Hsp90 under these conditions (Fig. 7B, lanes 4, 11 and 12). Most interestingly, we detected stable interaction between FLAG-EBNA-3 and Hsp90 in cells where non-tagged XAP2 was co-expressed (Fig. 7B, lane 2). This interaction, however, was abrogated by the presence of AhR (Fig. 7B, lanes 9 and 10). Ligand treatment had no effect on the interactions examined.

EBNA-3 Enhances the Transactivation Function of the AhR—The ability of EBNA-3 to interact with the ligand-activated AhR prompted us to examine the influence of EBNA-3 on the ligand-inducible transactivation function of the AhR. To this end, we performed reporter gene assays in MCF7 cells using a reporter plasmid construct that encodes the firefly luciferase gene driven by the minimal thymidine kinase promoter and two XRE motifs. The cells were transfected with increasing amounts of the FLAG-EBNA-3-expressing plasmid or empty vector (pCMV2/FLAG) in control cells. Following transfection, cells were treated with 10 nM TCDD or vehicle (Me2SO) alone for 24 h. The results in Fig. 8 show that, in the presence of EBNA-3, the ligand-induced transcriptional activity of AhR was increased by more than 30% compared with control cells. It is noteworthy that the increase in luciferase activity in control cells was observed in parallel with the increase of the transfected DNA material (the concentration of the reporter plasmid was kept the same). This general trend is because of the difference in transfection efficiency, which depends on the amount of the transfected DNA material.
DNA. Therefore, the observed positive effects by EBNA-3 on the trans-activation function of the AhR was not considered as dose-dependent in this experiment.

**EBNA-3 Counteracts TCDD-induced Growth Inhibition—Studies on murine B-cell development have shown that AhR ligands, such as polycyclic aromatic compounds, induce apoptosis in pro-B- and pre-B-cells** (28). In this study, we wanted to investigate the influence of TCDD on the proliferation rate of EBV-immortalized and EBV-negative B-cells. We tested the following four cell lines: freshly established LCLs (1 month after EBV infection), DG75 (EBV-negative Burkitt lymphoma line), and DG75 cells that constitutively expressed either EBNA-3 or EBNA-5. TCDD was added to the growth medium at a concentration of 10 nM for 16 days, with medium change every 3 days. As shown in Fig. 9, DG75 and DG75-EBNA-5 grew more slowly in the presence of TCDD. The growth of LCLs that express EBNA-3 and EBNA-3-expressing DG75 cells was unimpaired by TCDD treatment. These data suggest that binding of EBNA-3 to AhR antagonizes TCDD-induced inhibition of cell growth in EBV-infected B-cells.

**DISCUSSION**

We have previously detected an interaction between EBNA-3 and the AhR- and Hsp90-interacting protein XAP2 (10). In this study, we have documented specific binding of EBNA-3 to AhR. Most interestingly, the stability of the EBNA-3-AhR complex seems to be dependent on whether XAP2 is present in the AhR complex. This is indicated by the observation that XAP2 inhibited binding of EBNA-3 to the latent AhR, whereas EBNA-3 had no effect on AhR-XAP2 complex formation. These observations were corroborated by immunostaining experiments, in which XAP2-induced cytoplasmic retention of the AhR was dominant over co-expressed EBNA-3. However, in the presence of TCDD, binding of EBNA-3 to AhR was stabilized, although it remains unclear whether this was due to ligand-induced dissociation of XAP2, together with the chaperone Hsp90 complex, or a conformational change of the AhR upon ligand binding. Despite the presence of XAP2, known to markedly delay ligand-dependent nuclear import of the receptor (16), ligand-induced nuclear translocation of AhR occurred efficiently in the presence of EBNA-3. Most interestingly, AhR-free XAP2, when co-expressed with EBNA3, seems to “obey” the nuclear pattern of EBNA-3 by co-localizing within spot-like nuclear conglomerates. On the other hand, the formation of the EBNA-3-AhR complex leads to a re-organization of the subcellular localization of EBNA-3 because the nuclear conglomerate pattern, a characteristic feature of EBNA-3, became diffuse when co-expressed with AhR. It is also interesting to note that a fraction of EBNA-3 stained in the cytoplasm in cells expressing all three examined proteins, EBNA-3, AhR-GFP, and XAP2, resulting in the cytoplasmic co-localization with both AhR-GFP and XAP2. This suggests that intermediate ternary AhR-XAP2-EBNA-3 complexes may exist in the cytoplasm despite the negative effect of XAP2 upon binding of EBNA-3 to the receptor. These potential com-
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FIGURE 9. The influence of TCDD on lymphocyte proliferation in the absence or presence of EBNA-3. DG-75, DG-75-EBNA-3, and DG-75-EBNA-5 cell lines were grown in the presence of 10 nM TCDD or vehicle (Me2SO) alone. Cells were grown in Iscove's medium by diluting them (1:2 or 1:4) every 3rd day with fresh medium (− TCDD). Cells were counted at indicated times of growth. The number of TCDD-treated cells relative to the Me2SO-treated (control) cells are indicated in %.

FIGURE 10. Model of interaction of EBNA-3 with the AhR in EBV-transformed cells. The model shows interaction between EBNA-3 and the AhR during the ligand-induced AhR activation process and potentiation of the transactivation function of the activated receptor form. See text for details.

A possible interaction between EBNA-3 and the molecular chaperone Hsp90 provides another intriguing aspect on the functional interplay between EBNA-3 and the latent form of AhR. EBNA-3 does not seem to interact with Hsp90 on its own, but a ternary complex can be formed with this chaperone in the presence of XAP2. Most interestingly, the EBNA-3-XAP2-Hsp90 complex was disrupted in the presence of AhR. We were also unable to detect the interaction of EBNA-3 with AhR-bound Hsp90. These data prompt us to speculate that the stability of the EBNA-3-AhR complex depends on the strength of interaction between the AhR and Hsp90, which, in turn, depends upon the activation state of the receptor (Fig. 10). This model would explain the increase in the stability of the interaction between EBNA-3 and the ligand-activated AhR, which is free of Hsp90. On the other hand, XAP2 binding to the latent AhR stabilizes the association of the receptor with Hsp90 (27). This may provide an explanation why the interaction between EBNA-3 and AhR is unstable in the presence of XAP2. Furthermore, interaction of EBNA-3 with the ligand-bound AhR may facilitate the release of AhR and Hsp90, and, as the end result, up-regulate the transcriptional activity of AhR. However, the exact molecular mechanisms responsible for the positive effects of EBNA-3 upon the activation process of the AhR remain to be clarified.

What are the common features that could link EBV-induced lymphocyte transformation and the ubiquitous AhR-mediated signaling pathway, in addition to the fact that two proteins of these pathways, EBNA-3 and AhR, bind to the same protein, XAP2? A number of recent studies have shown that AhR and EBV-encoded proteins such as EBNA-3, LMP1, and EBNA-5 participate in cell cycle regulation (reviewed in Refs. 29 and 30). The molecular details of the AhR and EBNA-3 function in cell cycle regulation remain unknown. Studies using AhR-defective mouse hepatoma Hepa1c1c7 cell line variants have indicated that the passage of the cells through the G1 phase is delayed because of the lack of the functional AhR (31). In another study, mouse embryonic fibroblasts, derived from AhR-null mice, proliferate more slowly because of the accumulation of cells in the G1/M phase, possibly due to altered expression of two G1/M kinases, Cdc2 and Plk (32). These reports suggest that the nonactivated form of AhR may promote cell cycle progress. In contrast, TCDD-exposed rat hepatoma 5L cells undergo an arrest at G1. This TCDD-dependent negative effect on cell cycle progression was attributed largely to the increased expression of the CDK2 inhibitor p27Kip1 (33). The involvement of AhR in cell cycle control is also consistent with its interaction with proteins such as pRb (34) and the p65 subunit of NFKB RelA (35). It is noteworthy that AhR and RelA are known to affect each other in mutually repressive ways; AhR inhibits binding of NFKB to NFKB regulatory sites, whereas RelA suppresses TCDD-inducible promoters (35). On the other hand, AhR and RelA have been shown to cooperate in activating the c-myc promoter in breast cancer lines (36).

What could be the physiological consequences of the EBNA-3-AhR interaction? Despite the positive effects of EBNA-3 upon the AhR-mediated transactivation, we have found that in EBV-infected B-lymphocytes, EBNA-3 counteracts TCDD-induced cell growth inhibition. Thus, the effects of EBNA-3 upon the AhR-mediated signaling may be divided into “short term” effects (AhR activation) and “long term” effects (TCDD/AhR-induced cell growth). A plausible interpretation would suggest that EBV uses the EBNA-3-AhR interaction as a part of its growth transformation strategy, designed to divert TCDD-AhR-dependent negative effects on cell proliferation and survival. In this respect the interplay between EBV- and AhR-mediated effects in relation to the NFKB-mediated signaling pathway, known to play a vital role in blast transformation, should be examined. It is known that during the process of EBV-induced B-cell transformation, the LMP1 protein is involved in the activation of NFKB through its binding to TRAF1–3 (37). Whenever the AhR pathway is activated in lymphoid cells, the EBNA-3 protein might sequester AhR and thereby prevent the interaction between the AhR and NFKB, thus inhibiting the EBV-induced blast transformation.

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REFERENCES
1. Kieff, E., and Rickinson, A. (2001) in Fields Virology (Knipe, D. M., and Howley, P. M., eds) 4th Ed., Vol. II, pp. 2511–2574, Lippincott Williams & Wilkins, Philadelphia
2. Kuppers, R. (2003) Nat. Rev. Immunol. 3, 801–812
EBNA-3 Interacts with the AhR Complex

3. Cooper, A., Johannsen, E., Maruo, S., Cahir-McFarland, E., Illanes, D., Davidson, D., and Kieff, E. (2003) J. Virol. 77, 999–1010
4. Maruo, S., Johannsen, E., Illanes, D., Cooper, A., and Kieff, E. (2003) J. Virol. 77, 90437–10447
5. Robertson, E. S., Lin, J., and Kieff, E. (1996) J. Virol. 70, 3068–3074
6. Krauer, K. G., Kienzle, N., Young, D. B., and Sculley, T. B. (1996) Virology 226, 346–353
7. Zhao, B., Marshall, D. R., and Sample, C. E. (1996) J. Virol. 70, 4228–4236
8. Hickabottom, M., Parker, G. A., Freemont, P., Crook, T., and Allday, M. J. (2002) J. Biol. Chem. 277, 47197–47204
9. Kashuba, E., Kashuba, V., Sandalova, T., Klein, G., and Szekely, L. (2002) BMC Cell Biol. 3, 23
10. Kashuba, E., Kashuba, V., Pokrovskaja, K., Klein, G., and Szekely, L. (2000) Oncogene 19, 1801–1806
11. Carver, L. A., LaPres, J. J., Jain, S., Dunham, E. E., and Bradfield, C. A. (1998) J. Biol. Chem. 273, 33580–33587
12. Ma, Q., and Whitlock, J. P., Jr. (1997) J. Biol. Chem. 272, 8878–8884
13. Meyer, B. K., Pray-Grant, M. G., Vanden Heuvel, J. P., and Perdew, G. H. (1998) Mol. Cell. Biol. 18, 978–988
14. Gu, Y. Z., Hogenesch, J. B., and Bradfield, C. A. (2000) Annu. Rev. Pharmacol. Toxicol. 40, 519–561
15. Kazlauskas, A., Sundström, S., Poellinger, L., and Pongratz, I. (2001) Mol. Cell. Biol. 21, 2594–2607
16. Kazlauskas, A., Pongratz, I., and Poellinger, L. (2000) J. Biol. Chem. 275, 41317–41324
17. McGuire, J., Whitelaw, M. L., Pongratz, I., Gustafsson, J.-Å., and Poellinger, L. (1994) Mol. Cell. Biol. 14, 2438–2446
18. Poland, A., and Knutson, J. C. (1982) Annu. Rev. Pharmacol. Toxicol. 22, 517–554
19. Andersson, P., McGuire, J., Rubio, C., Gradin, K., Whitelaw, M. L., Pettersson, S., Hanberg, A., and Poellinger, L. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 9990–9995
20. Whitelaw, M. L., Pongratz, I., Wilhelmsson, A., Gustafsson, J.-Å., and Poellinger, L. (1993) Mol. Cell. Biol. 13, 2504–2514
21. Berghard, A., Gradin, K., Pongratz, I., Whitelaw, M., and Poellinger, L. (1993) Mol. Cell. Biol. 13, 677–689
22. Kashuba, E., Mattsson, K., Klein, G., and Szekely, L. (2003) Mol. Cancer 2, 18
23. Pokrovskaja, K., Mattsson, K., Kashuba, E., Klein, G., and Szekely, L. (2001) J. Gen. Virol. 82, 345–358
24. Kashuba, E., Pokrovskaja, K., Klein, G., and Szekely, L. (1999) J. Hum. Virol. 2, 33–37
25. Denis, M., Cuthill, S., Wikström, A.-C., Poellinger, L., and Gustafsson, J.-Å. (1988) Biochem. Biophys. Res. Commun. 155, 801–807
26. Isaguliants, M. G., Gudima, S. O., Ivanova, O. V., Levi, M., Hinkula, J., Garaev, M. M., Kochetkov, S. N., and Wahren, B. (2000) AIDS Res. Hum. Retroviruses 16, 1269–1280
27. Kazlauskas, A., Poellinger, L., and Pongratz, I. (2002) J. Biol. Chem. 277, 11795–11801
28. Yamaguchi, K., Near, R. I., Matulka, R. A., Schneider, A., Toselli, P., Trombino, A. F., and Sherr, D. H. (1997) J. Immunol. 158, 2165–2173
29. Murray, P. G., and Young, L. S. (2001) Exp. Rev. Mol. Med. 15, 1–20
30. Puga, A., Xia, Y., and Elferink, C. (2002) Chem. Biol. Int. 141, 117–130
31. Ma, Q., and Whitlock, J. P., Jr. (1996) Mol. Cell. Biol. 16, 2144–2150J. P.
32. Elizondo, G., Fernandez-Salguero, P., Sheikh, M. S., Kim, G. Y., Fornace, A. J., Lee, K. S., and Gonzalez, F. J. (2000) Mol. Pharmacol. 57, 1056–1063
33. Kolluri, S. K., Weiss, C., Koff, A., and Gottlichter, M. (1999) Genes Dev. 13, 1742–1753
34. Ge, N. L., and Elferink, C. J. (1998) J. Biol. Chem. 273, 22708–22713
35. Tian, Y., Ke, S., Denison, M. S., Rabson, A. B., and Gallo, M. A. (1999) J. Biol. Chem. 274, 510–515
36. Kim, D. W., Gazourian, L., Quadri, S. A., Romieu-Moure, R., Sherr, D. H., and Sonenshein, G. E. (2000) Oncogene 19, 5498–5506
37. Devergne, O., Hatzivassiliou, E., Iizumi, K. M., Kaye, K. M., Kleijnen, M. F., Kieff, E., and Mosialos, G. (1996) Mol. Cell. Biol. 16, 7098–7108