Epstein–Barr virus-encoded EBNA1 modulates the AP-1 transcription factor pathway in nasopharyngeal carcinoma cells and enhances angiogenesis in vitro

John D. O’Neil, Thomas J. Owen, Victoria H. J. Wood,† Kathryn L. Date, Robert Valentine, Marilyn B. Chukwuma, John R. Arrand, Christopher W. Dawson and Lawrence S. Young

Cancer Research UK Institute for Cancer Studies, University of Birmingham, Vincent Drive, Edgbaston, Birmingham B15 2TT, UK

The Epstein–Barr virus (EBV)-encoded EBNA1 protein is expressed in all virus-associated tumours, including nasopharyngeal carcinoma (NPC), where it plays an essential role in EBV genome maintenance, replication and transcription. Previous studies suggest that EBNA1 may have additional effects relevant to oncogenesis, including enhancement of cell survival, raising the possibility that EBNA1 may influence cellular gene expression. We have recently demonstrated by gene expression microarray profiling in an NPC cell model that EBNA1 influences the expression of a range of cellular genes, including those involved in transcription, translation and cell signalling. Here, we report for the first time that EBNA1 enhances activity of the AP-1 transcription factor in NPC cells and demonstrate that this is achieved by EBNA1 binding to the promoters of c-Jun and ATF2, enhancing their expression. In addition, we demonstrate elevated expression of the AP-1 targets interleukin 8, vascular endothelial growth factor (VEGF) and hypoxia-inducible factor-1α in response to EBNA1 expression, which enhances microtubule formation in an in vitro angiogenesis assay. Furthermore, we confirm elevation of VEGF and the phosphorylated isoforms of c-Jun and ATF2 in NPC biopsies. These findings implicate EBNA1 in the angiogenic process and suggest that this viral protein might directly contribute to the development and aggressively metastatic nature of NPC.

INTRODUCTION

Epstein–Barr virus (EBV) is a ubiquitous human gamma-herpesvirus associated with both lymphoid and epithelial tumours (Kieff & Rickinson, 2001). The pattern of EBV latent protein expression differs in these tumours, with the EBV nuclear antigen EBNA1 alone being expressed in Burkitt’s lymphoma (BL) while EBNA1 and two membrane proteins (LMP1 and LMP2A/B) are expressed in Hodgkin’s lymphoma (HL) and nasopharyngeal carcinoma (NPC) (Kieff & Rickinson, 2001; Young & Rickinson, 2004; Raab-Traub, 2002). The consistent expression of EBNA1 in all EBV-related malignancies is a result of the indispensable role that EBNA1 plays in maintenance and replication of the EBV genome via sequence-specific binding to the viral origin of replication, oriP (Raab-Traub, 2002). In addition to the role that EBNA1 plays in viral genome maintenance, it also interacts with viral gene promoters, thereby contributing to the transcriptional regulation of the EBNA3s and of LMP1. Recent studies have also demonstrated that EBNA1 can interact with the ubiquitin-specific protease USP7, which has been implicated in the stabilization of p53. Holowaty & Frappier (2004) showed that EBNA1 can bind with a higher affinity to the same region of USP7 as p53 and MDM2 and suggest that, as a consequence, EBNA1 can protect against either UV- or p53-induced apoptosis. A more direct involvement of EBNA1 in carcinogenesis has been suggested by the ability of B-cell-directed EBNA1 expression to produce B-cell lymphomas in transgenic mice (Wilson et al., 1996). However, dominant-negative EBNA1 (dnEBNA1) studies in a lymphoblastoid cell line with an integrated EBV genome revealed no effect of EBNA1 on cell growth or cellular gene expression (Kang et al., 2001). Furthermore, studies in which EBNA1 has been expressed in Akata BL cells previously cleared of EBV infection demonstrated that EBNA1 expression alone is not sufficient to confer tumorigenic potential (Komano et al., 1998; Ruf et al., 2000).

†Present address: Laboratory of Cancer Biology, University of Oxford, Room 1501, Women’s Centre, John Radcliffe Hospital, Headley Way, Oxford OX3 9DU, UK.

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Whilst EBNA1’s contribution to the development of EBV-related tumours remains controversial, the ability of EBNA1 to modulate viral gene expression suggested that it was likely that EBNA1 could also influence cellular gene expression. This has been demonstrated in the context of B cells, where EBNA1 has been shown to induce CD25 expression in an EBV-negative HL cell line and to upregulate RAG1 and RAG2 expression in a BL cell line (Srinivas & Sixbey, 1995; Kube et al., 1999). We have recently established that EBNA1 expression in a carcinoma cell line resulted in the upregulation of 113 cellular genes and the downregulation of 49 genes (Wood et al., 2007). Furthermore, validation of a number of these changes revealed that EBNA1 influences the expression of a range of cellular genes, including those involved in translation, transcription and cell signalling. We found that EBNA1 expression enhanced STAT1 expression, which sensitized cells to interferon-induced STAT1 activation and repressed the TGFβ1 signalling pathway. As these data revealed that EBNA1 can influence cellular gene transcription, resulting in effects that may contribute to the development of EBV-associated tumours such as NPC, we sought to determine whether EBNA1 could also modulate cellular gene expression by influencing other key transcriptional pathways, where aberrations have been implicated in oncogenesis.

**METHODS**

**Cell lines and tissue culture.** HEK293 (transformed normal human embryonic kidney cells), Ad/AH (a human adenocarcinoma cell line derived from the nasopharynx), HONE1 (an EBV-negative NPC cell line) and AGS (a human gastric-derived carcinoma cell line) cell lines were cultured in RPMI medium 1640 supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine and 1% penicillin–streptomycin solution (Sigma-Aldrich). Ad/AH, HONE1 and AGS cells stably expressing EBNA1 and the Ad/AH line stably infected with a recombinant EBV were generated as described previously (Wood et al., 2007), HMEC-1 cells (Cancer Research UK cell line repository) were cultured in Biocoat Endothelial Cell Growth Environment medium (BD Biosciences) amended with 8% FCS and 1% penicillin–streptomycin. Cultured in Biocoat Endothelial Cell Growth Environment medium (BD Biosciences) was harvested from Ad/AH cells cultured in RPMI 1640 supplemented with 0.5% FCS, 2 mM L-glutamine and 1% penicillin–streptomycin solution (Sigma-Aldrich). Ad/AH, HONE1 and AGS cells stably expressing EBNA1 and the Ad/AH line stably infected with a recombinant EBV were generated as described previously (Wood et al., 2007), HMEC-1 cells (Cancer Research UK cell line repository) were cultured in Biocoat Endothelial Cell Growth Environment medium (BD Biosciences) amended with 8% FCS and 1% penicillin–streptomycin. All cells were cultured at 37 °C with 5% CO₂ and 20% O₂, unless stated otherwise. Hypoxic growth conditions were achieved by incubating cells at 37 °C with 5% CO₂ and 1% O₂ for 24 h.

**Luciferase assays and transient transfection.** Dual luciferase reporter assays were performed according to manufacturer’s instructions (Promega) with cells cultured in RPMI 1640 supplemented with 0.5% FCS, 2 mM L-glutamine, and 1% penicillin–streptomycin solution (Sigma-Aldrich) throughout. Cells were transfected with the following plasmids using Lipofectamine (Invitrogen) following the manufacturer’s instructions: pSG5-EBNA1 (Sample et al., 1992), pSG5-LMP1 (Eliopoulos et al., 1997), pG3-basic (Promega), AP-1-luciferase reporter (BD Biosciences), interleukin (IL)-8-luc and IL-8-mut reporters (Eliopoulos et al., 1999), c-Jun promoter luciferase reporter (Wei et al., 1998), AP-1 wild-type and mutant decoy oligonucleotides (Ahn et al., 2002), dnEBNA1 (Marchal et al., 1999), dominant-negative c-Jun (dn-c-Jun) (Ham et al., 1995) and a control Renilla luciferase plasmid (pRL-TK; Promega). 12-O-tetradecanoyl-phorbol-acetate (TPA) stimulation was achieved by the addition of 60 ng TPA ml⁻¹ for 16 h prior to harvesting. Endothelial growth factor (EGF) stimulation was achieved by the addition of 100 ng EGF ml⁻¹ for 3 h.

All assays were carried out in biological and technical triplicate and are represented as the mean of three independent experiments.

**Quantitative RT-PCR (qRT-PCR) and immunoblotting analysis.** RNA was extracted using EZ-RNA total RNA isolation kit (Geneflow) and amplification by qRT-PCR was performed using an ABI 7500 real-time PCR machine following standard procedures. qRT-PCR Taqman primer and probe sets for c-Jun, ATF2 and IL-8 were purchased from Applied Biosystems (Hs00999914_s1, Hs00153179_m1 and Hs00174103_m1, respectively). Taqman hydroxide inducible factor (HIF)-1α primers and probe set were as follows: 5'-ATGAAC-ATAAAAGCTGTGACATGGA-3' (forward), 5’-CTGAGGTTGTGTT-ACGTTGCTATCATATA-3' (reverse) and 5’-TGACAGCTGACAG-GCCACATTTCAC-3' (probe). Standard immunoblotting procedures (Young & Rickinson, 2004) were used to detect HIF-1α (1 μg mouse MAB1536 ml⁻¹; R&D Systems).

**TransAM analysis.** Nuclear protein extracts were isolated following the manufacturer’s instruction (Active Motif). The AP-1 subunits present in active AP-1 dimers were measured using the ELISA based TransAM AP-1 family and ATF2 kit (Active Motif) according to the manufacturer’s instruction.

**Cytokine analysis.** ELISA analysis for secreted IL-8 and vascular endothelial growth factor (VEGF) was performed following the manufacturers’ instructions (Sanquin Reagents and R&D Systems, respectively).

**Electrophoretic mobility shift assay (EMSA).** Nuclear extracts were prepared and EMSA analysis was carried out on 5 μg nuclear protein according to the manufacturer’s instructions (Li-Cor Biosciences) using a wild-type AP-1 probe (sense oligonucleotide 5’-IRDye700-CGCTTGTAGACTCAAGCGGAA-3’) and a mutant AP-1 probe (sense oligonucleotide 5’-IRDye700-CGCTTGTAGACTCAAGCGGAA-3’); nucleotides in bold type indicate the AP-1 binding motif. EMSA gels were analysed and images were captured using the Li-COR Odyssey infrared laser imaging system. EMSAs were repeated for three independent biological replicates.

**Chromatin immunoprecipitation (ChIP) assays.** ChIP assays were performed following the protocol provided by Upstate Biotechnology (catalogue no. 17-371), using an EBNA1 antibody (chEBNA1), the conditions described by Chau & Lieberman (2004) and a rabbit isotype control antibody (Santa Cruz). Real-time quantitative PCR was performed on ChIP DNA using the Taqman primers and probe sets detailed in Supplementary Table S1 (available in JGV Online) using an ABI 7500 real-time PCR machine. Statistical significance was determined using Student’s t-test.

**In vitro microtubule formation assay.** Conditioned growth medium was harvested from Ad/AH cells cultured in RPMI 1640 supplemented with 0.5% FCS, 2 mM L-glutamine and 1% penicillin–streptomycin solution (Sigma-Aldrich) for 48 h. The microtubule formation assay was performed using HMEC-1 cells resuspended in control (RPMI 1640 supplemented with 5% FCS, 2 mM L-glutamine and 1% penicillin–streptomycin solution) or conditioned growth media layered onto Matrigel according to the manufacturer’s instructions (BioCoat Endothelial Cell Tube Formation Angiogenesis System; BD Biosciences). Images were captured after 6–10 h of incubation at 37 °C with 5% CO₂ and 20% O₂. The number and length of tubes were determined using Axiovision image analysis software (Zeiss Imaging Solutions). At least 100 tubes were measured over at least three fields of view. Statistical significance was determined using Student’s t-test.

**Immunohistochemistry.** Sections of paraffin-embedded Chinese NPC biopsies were processed using the agitated low temperature
epitope retrieval (ALTER) method (Hussain et al., 2007), using the following primary antibodies: p-c-Jun (mouse monoclonal SC-822, 1:100; Santa Cruz), p-ATF2 (mouse monoclonal sc-8398, 1:100; Santa Cruz) and VEGF (mouse monoclonal VG-1, 1:100; Cancer Research UK). Sections were counterstained with haematoxylin.

RESULTS

EBNA1 enhances AP-1 activity in epithelial cells

The promoters of genes found to be differentially regulated by EBNA1 in Ad/AH cells (commonly used as an NPC cell model) using microarray analysis (Wood et al., 2007; unpublished data) were screened for host cell transcription factor DNA-binding motifs using the promoter analysis and interaction network toolset (PAINT) (Vadigepalli et al., 2003). This in silico approach revealed that 20% (495 of 2453) of the promoters of cellular genes differentially regulated by EBNA1 contained AP-1-binding sites, including the promoters of IL-8 and VEGF (data not shown). As it had been reported that functional homologues of EBNA1 from Kaposi’s sarcoma-associated herpesvirus (KSHV) latency-associated nuclear antigen (LANA) and human papillomavirus (HPV) (E2) (An et al., 2002; Behren et al., 2005) modulate AP-1 activity, we set out to determine whether the ability of EBNA1 to influence cellular gene expression was mediated by the AP-1 transcription factor. Initially, we performed luciferase reporter assays in a range of epithelial cells using a synthetic AP-1 reporter. Transient expression of EBNA1 in HEK293, Ad/AH, HONE1 and AGS cells resulted in an increase in AP-1 activity that was comparable to that achieved by TPA stimulation or transfection with LMP1 (Fig. 1a). To assess whether enhanced AP-1 activity required a functional EBNA1, dnEBNA1 was titrated against wild-type EBNA1 expressed at a concentration comparable to that seen during EBV infection in Ad/AH cells, and AP-1 luciferase activity was measured. Increasing doses of dnEBNA1 resulted in almost complete abrogation of the ability of wild-type EBNA1 to enhance AP-1 activity (Fig. 1b). Expression of dnEBNA1 alone did not result in enhanced AP-1 activity (Supplementary Fig. S1).

As transient EBNA1 expression resulted in enhanced AP-1 activity, we set out to determine whether this observation could be confirmed in the stable EBNA1-expressing Ad/AH cells used previously in our microarray analysis. A 2.3-fold enhancement of AP-1 activity was seen in the Ad/AH cells stably expressing EBNA1; the ability of stable EBNA1 expression to enhance AP-1 activity was abrogated by transfection with AP-1 decoy oligonucleotides designed to sequester transcriptionally competent AP-1 (Fig. 1c). Furthermore, AP-1 activity was assayed in two additional

Fig. 1. EBNA1 enhances AP-1 activity in epithelial cells. Luciferase reporter assay following transient transfection of HEK293, Ad/AH, HONE1 and AGS cells with an AP-1 luciferase reporter plasmid and increasing concentrations of an EBNA1 expression plasmid (pSG5-EBNA1) (a) or a constant amount of an EBNA1 plasmid and increasing concentrations of a dnEBNA1 plasmid (b). In (a), TPA treatment (60 ng ml$^{-1}$ for 16 h) and transfection of an LMP1 plasmid (pSG5-LMP1) served as the assay positive controls and empty vector (zero EBNA1 expression) served as the negative control. (c) Transient transfection of Ad/AH cells stably expressing either EBNA1 or an empty vector control plasmid (Neo) with an AP-1 luciferase reporter plasmid and varying ratios of wild-type to mutant AP-1 decoy oligonucleotides. (d) EMSA analysis of equal amounts of nuclear protein from Ad/AH, HONE1 and AGS cells stably expressing either EBNA1 (E1), an empty vector control plasmid (Neo) or empty vector control cells stimulated with 60 ng TPA ml$^{-1}$ for 16 h. Nuclear protein (5 μg) was incubated with either an IRDye700-conjugated wild-type AP-1 DNA probe or an IRDye700-conjugated mutant AP-1 probe (Mut). Results are means ± SD derived from three biological replicates, each performed in technical triplicate.
Ad/AH stable EBNA1 clones, yielding results comparable to those presented here (Supplementary Fig. S2, available in JGV Online). In addition, EMSA analysis demonstrated enhanced binding of an AP-1-specific DNA probe with nuclear protein from stable EBNA1-expressing Ad/AH, HONE1 and AGS cells. The degree of enhanced DNA binding in the EBNA1 expressing cells was comparable to that seen upon TPA stimulation of the Neo control cells. In contrast, a mutant AP-1 probe resulted in almost undetectable levels of DNA binding, which demonstrated the high degree of specificity within the assay (Fig. 1d).

**EBNA1 enhances IL-8 promoter activity and IL-8 expression in an AP-1-dependent manner in Ad/AH cells**

The ability of EBNA1 to enhance AP-1 activity prompted us to examine whether this resulted in changes in cellular gene expression. As microarray analysis had identified upregulation of IL-8, a well-documented target of AP-1, in EBNA1-expressing Ad/AH cells, we performed IL-8 luciferase assays and qRT-PCR to determine whether EBNA1 expression influenced IL-8 expression in an AP-1-dependent manner. Transfection of the stable EBNA1-expressing Ad/AH cells and their Neo counterpart with a wild-type IL-8 luciferase reporter construct revealed a 2.5-fold increase in reporter activity in the EBNA1 cells (Fig. 2a). Furthermore, the ability of EBNA1 to elevate IL-8 reporter activity was attenuated by deletion of the AP-1 site (Fig. 2a). The ability of EBNA1 to elevate IL-8 expression was confirmed using qRT-PCR in Ad/AH cells transiently transfected with increasing doses of EBNA1 (to a maximum of approximately threefold) as well as in Ad/AH cells stably expressing EBNA1 (Fig. 2b). In addition, abrogation of AP-1 activity by transfecting Ad/AH cells with an EBNA1 plasmid and increasing doses of dnc-Jun plasmid demonstrated that the ability of EBNA1 to induce IL-8 transcription was significantly reduced by high doses of dnc-Jun (Fig. 2c).

**EBNA1 enhances the secretion of the angiogenic cytokines IL-8 and VEGF**

In light of our evidence that EBNA1 elevated IL-8 promoter activity and transcription in an AP-1-dependent manner, and that elevated IL-8 expression and secretion have been reported to contribute to angiogenesis and the aggressive metastasis seen in NPC (Yoshizaki et al., 2001), we sought to determine whether EBNA1 could contribute...
to the secretion of angiogenic cytokines, many of which are regulated by AP-1. ELISA demonstrated that IL-8 secretion was elevated by 1.6-fold and VEGF was elevated by 1.4-fold in the Ad/AH cells stably expressing EBNA1, with a statistical significance of \( P < 0.05 \) (Fig. 2d). Furthermore, there was a significant \( (P < 0.05) \) increase in the secretion of IL-8 and VEGF (2.8- and 1.9-fold, respectively) in Ad/AH cells stably infected with recombinant EBV.

**EBNA1 enhances the expression of the \( \alpha \) subunit of HIF-1**

The major stimulus that initiates angiogenesis and neovascularization of solid tumours is hypoxia, and a key transcription factor that orchestrates angiogenesis in response to hypoxia is HIF-1 \( (\text{Choi et al., 2003}) \). We had demonstrated that EBNA1 enhanced the secretion of IL-8 and VEGF, both of which are transcriptional targets of HIF-1, and our microarray data indicated that the HIF-1 subunit HIF-1\( \alpha \), a transcriptional target of AP-1, was upregulated in Ad/AH EBNA1 cells. Therefore, we examined whether EBNA1 expression impacted upon the expression of HIF-1\( \alpha \). qRT-PCR demonstrated that expression of HIF-1\( \alpha \) was enhanced in both the EBNA1-expressing cells and Ad/AH cells stably infected with EBV in response to hypoxic growth conditions (2.25- and 2.36-fold, respectively) (Fig. 3a). Immunoblot analysis validated these findings at the protein level and also indicated that the phenomenon was abrogated by transfection with dnc-Jun (Fig. 3b).

**EBNA1 enhances expression and activation of subunits of the AP-1 complex**

To gain insight into how EBNA1 could be enhancing AP-1 activity, we used qRT-PCR to examine the expression of two key AP-1 subunits, c-Jun and ATF2, and revealed that stable EBNA1 expression enhanced their transcription (fourfold and 2.4-fold, respectively) (Fig. 4a). In addition we found that total ATF2 protein was elevated by approximately fourfold in the EBNA1-expressing Ad/AH cells (Supplementary Fig. S3, available in JGV Online). In order to determine whether EBNA1 expression influenced the dimer composition of transcriptionally competent AP-1, we used TransAM analysis, which revealed that stable EBNA1 expression in Ad/AH cells resulted in an increase in the amount of phosphorylated c-Jun, JunB, JunD, FosB and ATF2 present in AP-1 dimers bound to the AP-1 probe (a 1.6-, 1.5-, 1.2-, 1.4- and 1.58-fold increase relative to the Neo control cells, respectively) (Fig. 4b). The enrichment of active AP-1 subunits afforded by EBNA1 expression was not observed using a mutated AP-1 probe (data not shown). Furthermore, ChIP analysis using an EBNA1-specific antibody revealed a 4.8- and 7.6-fold and statistically significant enrichment of c-Jun and ATF2 promoter DNA, respectively, relative to the isotype antibody control, indicating that EBNA1 was present at the c-Jun and ATF2 promoters in cells stably expressing EBNA1 (Fig. 4c). No significant enrichment was observed using ChIP qPCR oligonucleotides specific for the GAPDH promoter (negative control) or for the AP-1 subunits JunB, JunD, c-Fos, FosB, Fra1 or Fra2. In addition, transfection of Ad/AH cells stably expressing EBNA1 with a wild-type c-Jun promoter luciferase reporter indicated enhanced reporter activity comparable to that seen upon stimulation of the Neo control cells with EGF (positive control) (Fig. 4d).

**EBNA1 enhances microtubule formation**

Having demonstrated that stable EBNA1 expression in Ad/AH cells enhanced the secretion of IL-8 and VEGF and the transcription of HIF-1\( \alpha \), we wanted to assess whether EBNA1 could contribute to neovascularization in this
carcinoma cell model. We therefore assayed the ability of endothelial cells (HMEC-1) to form microtubules in Matrigel when cultured in control medium (5% FCS as an inducer of microtubule formation) or reduced serum growth medium (0.5% FCS) harvested from cells after 48 h of incubation (conditioned growth medium). We observed that conditioned growth medium from EBNA1-expressing cells (Fig. 5a, bottom left) stimulated the formation of a more comprehensive network of microtubules when compared with the conditioned medium harvested from Neo control cells (Fig. 5a, top left). Furthermore, the degree of microtubule formation approached that which was seen using conditioned growth medium from Ad/AH cells stably infected with EBV (Fig. 5a, bottom right) or the 5% FCS control (Fig. 5a, top right).

The degree of microtubule formation was determined from three separate fields of view for each conditioned growth medium or control by using computer-aided image analysis (Fig. 5b). The mean tube length was significantly greater in the control 5% FCS medium compared with the conditioned medium from the Neo control cells. Conditioned media from both EBV-infected and EBNA1-transfected Ad/AH cells resulted in a significant increase in mean tube length compared with the Neo control, reaching levels similar to that of the FCS control.

**VEGF, p-c-Jun and p-ATF2 are elevated in vivo in NPC biopsies**

The observation that EBNA1 enhanced the expression of c-Jun and ATF2 in Ad/AH cells and that AP-1 dimers bound to DNA were enriched for the phosphorylated and therefore transcriptionally active isoforms of these subunits led us to examine the expression of p-c-Jun and p-ATF2 in NPC biopsies. In addition, we chose to validate VEGF expression in NPC biopsies, as we had demonstrated that EBNA1 enhanced VEGF secretion in Ad/AH cells. Strong staining in tumour cells was observed in 12 of 19 cases (63%) for p-c-Jun, 7 of 20 cases (35%) for p-ATF2 and 12 of 24 cases (50%) for VEGF, compared with weak/absent staining in the surrounding infiltrate and stroma. Representative examples of the NPC biopsies studied are given in Fig. 6.
Here, for the first time, we have demonstrated enhanced AP-1 luciferase activity in several epithelial cell lines in response to EBNA1 expression. Furthermore, the enhancement of AP-1 activity in Ad/AH cells stably expressing EBNA1 was not confined to a single EBNA1 clone, confirming that the phenomenon was not merely due to clonal variation. The increase in AP-1 activity afforded by EBNA1 was considered to be substantial as it was comparable to that seen upon TPA stimulation (a potent activator of the AP-1 pathway) and separately by transfection with a plasmid expressing LMP1 (an EBV-encoded protein known to activate the AP-1 pathway). The observation that EBNA1 expression not only enhanced AP-1 luciferase activity but also led to enhanced AP-1 EMSA probe binding in multiple cell lines demonstrated that the ability of EBNA1 to enhance AP-1 activity was not restricted to an individual epithelial cell line or EBNA1-expressing isolate and suggested that the phenomenon could be applicable to epithelial cells in general. The possibility that AP-1 activity was elevated due to EBNA1 causing a general cellular stress response was addressed by transfecting EBNA1-expressing Ad/AH cells with a dnEBNA1 plasmid. This demonstrated that the ability of EBNA1 to enhance AP-1 activity was dependent on domains required for the trans-activation of viral genes and maintenance of the viral episome.

Whilst the classical view is that elevated AP-1 activity is oncogenic, the roles that individual AP-1 subunits play in tumorigenesis are complex, as different dimer combinations influence key cell fate decisions and can be considered as anti-oncogenic or oncogenic in a stimulus- and cell context-specific manner (Eferl & Wagner, 2003). Nevertheless, studies in mice and humans have allowed generalizations to be made regarding the transcriptional potency and relative contributions of dimer combinations to tumorigenesis (Eferl & Wagner, 2003; Hess et al., 2004). We found that stable EBNA1 expression in the Ad/AH cell line resulted in enhanced transcription of c-Jun and ATF2, that active AP-1 dimers bound to AP-1 probes were enriched for the phosphorylated and therefore active forms of c-Jun and ATF2, demonstrated by ChIP assays showing that EBNA1 was present at the promoters of c-Jun and ATF2, and that EBNA1 enhanced the c-Jun promoter luciferase activity. In addition, we observed elevated levels of phosphorylated AP-1 subunits JunB, JunD and FosB in the EBNA1 stable cell line. However, as ChIP analysis revealed that JunB, JunD and FosB promoter DNA was not statistically significantly enriched in EBNA1-expressing cells (though there was a non-significant fourfold enrichment of FosB promoter DNA) it was considered that the regulation of these subunits was likely to be more complex. Therefore, the influence of EBNA1 on the expression or activation of these subunits via mechanisms other than binding at their promoters, for example as a result of the EBNA1-mediated enhancement of general AP-1 activity impacting upon their expression, warrants further investigation. In agreement with our TransAM data, EBNA1 was found not to be present at the promoters of c-Fos, Fra1 or Fra2, which correlated with a lack of enrichment for the transcriptionally active isoforms of these subunits. In conclusion, EBNA1 modulates the AP-1 pathway in NPC cells by enhancing microtubule formation and activating the AP-1 pathway.
addition to the *in vitro* study, we report strong staining for p-c-Jun in NPC tumour cells from biopsy samples, which is in agreement with recently published observations (Tsai *et al.*, 2006) and, for the first time, we demonstrate strong staining for p-ATF2 in NPC tumour cells. The inability to detect p-ATF2 in all NPC cases may reflect methodological problems (e.g. antigen retrieval, antibody detection sensitivity) or variability between cases that may be relevant to tumour stage and grade.

Though we have not elucidated whether EBNA1 binds directly with promoter DNA or indirectly by associating with other proteins, we nevertheless propose that this promoter binding is the mechanism by which EBNA1 enhances the transcription of these two AP-1 subunits, resulting in elevated AP-1 activity in the NPC cell model studied. However, whilst we observed a correlation between EBNA1 binding at the ATF2 promoter and elevated expression of ATF2 mRNA and protein, it is difficult to draw comparisons between the degree of promoter DNA enrichment and the level of ATF2 expression, as promoter occupancy does not necessarily directly correlate with the magnitude of the resulting changes in gene expression. Similarly, it is difficult to draw comparisons between the strength of EBNA1 binding at the promoter of c-Jun and the resulting enhancement of mRNA expression or reporter activity. It is tempting to draw parallels between the ability of both LMP1 (Eliopoulos *et al.*, 1999) and EBNA1 to enhance the expression and activation of the key AP-1 subunits c-Jun and ATF2, particularly as these components exhibit strong DNA-binding affinity and potent oncogenic potential. Given that JunB, JunD and FosB have all generally been

**Fig. 6.** VEGF, p-c-Jun and p-ATF2 are elevated in NPC biopsies. Immunohistochemistry demonstrating *in vivo* expression of p-c-Jun, p-ATF2 and VEGF in paraffin sections of Chinese NPC biopsies. The black box in the left hand column (×200 magnification) indicates the field of view displayed in the right hand column (×400 magnification). Black closed arrows, NPC tumour cells. Black open arrows, cells of the surrounding infiltrate.
characterized as being antagonists of the oncogenic potential of c-Jun, an understanding of the precise effects of EBNA1 on the balance of AP-1 activity is essential (Eferl & Wagner, 2003).

In order to alleviate the hypoxic conditions commonly found in solid tumours, such as NPC, the oxygen deficit stimulates neovascularization by inducing the release of angiogenic cytokines – a process that is orchestrated by regulating the expression and activity of the α-subunit of the HIF-1 transcription factor (Minet et al., 1999; Salceda & Caro, 1997). We demonstrate here that EBNA1 specifically enhances both IL-8 promoter activity and expression in Ad/AH cells. Elevated IL-8 expression has been correlated with enhanced angiogenesis and metastasis and thus a generally more aggressive tumour phenotype in a wide range of solid tumours, including NPC (Yoshizaki et al., 2001; Gokhale et al., 2005). In addition, and in agreement with published data, we have demonstrated strong positive staining for VEGF in a high proportion (50%) of NPC cases studied (Krishna et al., 2006). Elevated VEGF expression and secretion have been reported to be associated with enhanced angiogenesis and poor prognosis both in NPC cell models and in NPC biopsies (Yoshizaki et al., 2001; Krishna et al., 2006; Qian et al., 2000). Moreover, the regulation of VEGF expression is controlled, at least in part, by AP-1 (Josko & Mazeuk, 2004).

The promoter of HIF-1α contains AP-1/ATF2-binding sites, and hypoxia-induced HIF-1 activity has been shown to enhance the expression of IL-8 and VEGF in carcinoma cells (Minet et al., 1999; Choi et al., 2003; Wakisaka et al., 2004). We found that the expression of HIF-1α RNA and protein is elevated by both EBNA1 expression and EBV infection in Ad/AH cells, an effect that is enhanced by hypoxia. This is in agreement with published data reporting that the HIF-1α protein is elevated in NPC (Chan et al., 2007; Wakisaka & Pagano, 2003) and that HIF-1α transcription is elevated in approximately 50% of gastric cancers, which correlated with VEGF-induced angiogenesis (Ma et al., 2007). In addition, abrogation of the ability of EBNA1 to enhance HIF-1α protein expression using dnc-Jun indicates that HIF-1α expression is regulated by AP-1 in the NPC cell model used in this study. Interestingly, it has recently been demonstrated that KSHV LANA, a functional homologue of EBNA1, interacts directly with the HIF-1α protein, resulting in its nuclear localization in U2OS cells (Cai et al., 2007). Furthermore, LANA leads to accumulation of HIF-1α in B lymphoma tumour cells latently infected with KSHV, by targeting VHL and p53 (both HIF-1α suppressors) for degradation (Cai et al., 2006). Our findings that EBNA1 not only enhances the expression of IL-8, VEGF and HIF-1α but also stimulates enhanced microtubule formation in an in vitro angiogenesis assay adds credence to our hypothesis that EBNA1 plays a role in angiogenesis, and that this role is multifaceted and could be of greater significance in the natural tumour setting where hypoxic conditions are prevalent.

In light of these observations, we are currently elucidating the exact composition(s) of AP-1 dimers in the Ad/AH cell line and other NPC cell lines. Furthermore, the ability of multiple EBV latent genes to impact upon AP-1 regulation and transcriptional activity suggests that targeting the AP-1 pathway could be of therapeutic value in the treatment of NPC.

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REFERENCES


