Short communication

Mutational analysis of the Epstein–Barr virus nuclear antigen 2 by far-Western blotting and DNA-binding studies

Christian Sauder,1 Nicole Götzinger,1 William H. Schubach,2 Gary C. Horvath,3 Elisabeth Kremmer,4 Alexander Krebs,1 Sigrid König,1 Ursula Zimber-Strobl,5 Nikolaus Mueller-Lantzsch1 and Friedrich A. Grässer1*

1 Institut für medizinische Mikrobiologie und Hygiene der Universitätshämatologikum, Haus 47, 66421 Hamburg/Saar, Germany, 2 Department of Medicine, University of Washington and Veterans Administration Medical Center, Seattle, Washington, USA, 3 Division of Oncology, Department of Medicine, HSC T-17, 080, State University of New York Stony Brook, NY 11790-8174, USA, 4 Institut für Immunologie, Hämatologikum, GSF, Marchioninistrasse 25, 81377 München, Germany and 5 Institut für Klinische Molekularbiologie und Tumorgenetik, Hämatologikum, GSF, Marchioninistrasse 25, 81377 München, Germany

We have previously shown by far-Western blotting that the Epstein–Barr virus nuclear antigen 2 (EBNA-2) both binds to a cellular protein of 130 kDa and histone H1, with the complex between EBNA-2 and p130 being tighter than between EBNA-2 and histone H1. Here we demonstrate that the N terminus of EBNA-2, which was previously shown to be necessary for transformation of B lymphocytes by EBNA-2, is essential for binding to p130. We further show data indicating that the binding of EBNA-2 to histone H1 appears not to be mediated exclusively via the basic Arg-Gly rich region in the C-terminal part of EBNA-2. With a MAb directed against the Trp-Trp-Pro (WWP) motif of EBNA-2, which is known to be essential for the interaction of EBNA-2 with the cellular factor RBPJκ/CBF1, we could inhibit the DNA binding of EBNA-2, providing further evidence that this region of EBNA-2 forms direct contact with RBPJκ/CBF1.

Epstein–Barr virus (EBV) is associated with the development of several human malignancies, including the endemic form of Burkitt’s lymphoma (BL), nasopharyngeal carcinoma and Hodgkin’s disease (for review see Miller, 1990). EBV is the causative agent of infectious mononucleosis and can induce acute lymphoproliferative disorders in immunocompromised or genetically susceptible humans. The infection of B lymphocytes by EBV in vitro leads to the outgrowth of permanently proliferating cell lines; in these latently infected cells only 10 virus genes are expressed. Of these, the five nuclear antigens EBNA-1, EBNA-2, EBNA-3A, EBNA-3C, EBNA-LP and the membrane protein LMP1 are essential for B cell immortalization by EBV (for review see Ring, 1994). EBNA-2 plays a decisive role in this process (Hammerschmidt & Sugden, 1989) and is one of the first genes to be expressed during immortalization by EBV. EBNA-2 activates transcription of the CD21, CD23 and c-fgr genes, it upregulates the expression of the virus TP1, TP2 and LMP1 genes and stimulates transcription from the virus BamHI C promoter (for review see Middleton et al., 1991). Several studies recently revealed that EBNA-2 exerts its transactivational function indirectly, mainly by interacting with the cellular DNA-binding protein RBPJκ, which was found to bind to its recognition sequence in the EBNA-2 response elements of the LMP1, CD23, TP1 and BamHI C promoters (Zimber-Strobl et al., 1994; Henkel et al., 1994; Ling et al., 1994). EBNA-2 interacts with RBPJκ both in the presence and absence of a DNA-binding site and it is suggested that RBPJκ tethers EBNA-2 to its response elements (Hsieh & Hayward, 1995). The transcription factor Spi-1/PU.1 was shown to bind to an EBNA-2 response element in the LMP1 promoter thus conferring EBNA-2 responsiveness to this promoter (Laux et al., 1994). Recently it was shown that the EBNA-2 C-terminal acidic domain is able to interact with TFIIB, TFIIF, TAF40 and a novel nuclear protein, p100 (Tong et al., 1995).

Using the far-Western blotting technique, we showed that EBNA-2 is able to interact with histone H1 (Grässer et al., 1993). Furthermore, we identified a strong
Fig. 1. (a) Schematic diagram of EBNA-2 and the various EBNA-2 mutants and summary of the results of DNA-binding and far-Western blot analyses. Numbers at the top refer to the EBNA-2 amino acid sequence of the strain M-ABA. P, polyproline domain; Divergent, sequence between EBNA-2A and EBNA-2B; RG, Arg-Gly repeat motif; neg, negatively charged domain. Solid bars indicate the length of the inserted linker. The sites of the point mutants are indicated by vertical bars. Hc24AH37, amino acids 1–197 deleted; H20AH28, d148–336; H37AH21, d197–322; ASph, d246–381; ANco, d322–369; H28AA63, d336–467; KT3, the nine C-terminal amino acids of EBNA-2 were replaced by the 11 C-terminal amino acids of the simian virus 40 large T antigen (KPPTPPPEPET); ANcoKT3, combination of ANco and KT3; Hae21, linker insertion (LE) at position 322 within the Trp-Trp322-Pro motif; SC, Cys388 replaced by serine; CS, Cys410 replaced by serine; SS, both cysteines replaced by serines. ND, Not determined. (b) Western-blot analysis (see Billaud et al., 1989 for description) of some of the EBNA-2 mutants expressed in the baculovirus system. The recombinant baculovirus expressing full-length EBNA-2 was described previously (Grässer et al., 1993). Recombinant
association of EBNA-2 with a 130 kDa protein and suggested that p130 plays a role in the EBNA-2-mediated transformation process (Grässer et al., 1993). At the beginning of our studies the protein responsible for targeting EBNA-2 to its promoter response elements had not been identified and we therefore wanted to know whether p130 was involved in DNA binding. To address this question we tried to identify the regions of EBNA-2 responsible for interaction with the promoter-binding protein and with p130. Moreover we wanted to investigate the role of cysteine residues 326 and 410 of EBNA-2 in DNA binding.

A series of deletion, linker insertion and point mutations of EBNA-2 was created as summarized in Fig. 1 (a). If p130 is involved in DNA binding and transformation, one of the four regions of EBNA-2 previously determined by Cohen et al. (1991) to be essential in terms of transformation and transactivation should also be necessary for binding to p130. Thus, the linker insertion mutants Hae21 and ANco, which are almost identical to mutants T1EBNA-2I320 and T1EBNA-2Δ321-378 (Cohen et al., 1991), were included in our study. These latter mutants have previously been shown to be non-transforming and not transactivating (Cohen et al., 1991). All of the mutants were expressed in vitro by transcription–translation to test for their ability to bind indirectly to the TP1 promoter in an electrophoretic mobility shift assay (EMSA). Deletion and insertion mutants spanning almost all of the EBNA-2-coding region were expressed in the baculovirus system to test for their ability to bind to p130 and histone H1 in far-Western blotting. Details about the construction of the mutants and the vectors for expression in vitro and by baculovirus can be obtained from the authors upon request. Western blot analysis of the mutants expressed in insect cells is shown in Fig. 1 (b). MAb R3 recognizes a C-terminal epitope which is deleted in the C-terminal mutant H28ΔA63. To detect this mutant, MAb 6C8 was raised against a peptide containing the EBNA-2 Trp-TrpPro (WWP) motif (Fig. 1), because this motif was shown to be necessary for transfection and binding to RBPJ, (Cohen et al., 1991; Ling & Hayward, 1995). We analysed the mutants for their ability to bind to the 130 kDa protein and histone H1 in far-Western blot analysis as shown in Fig. 2. Our results revealed that deletions in the C-terminal and central parts of EBNA-2 (mutants H28ΔA63, H37AH21 and ΔNcoKT3, respectively) had no effect on the ability of EBNA-2 to bind to p130 whereas deletion of the N-terminal amino acids 18 to 197 (mutant H24AH37) resulted in an almost complete loss of binding (Fig. 2a). The linker-insertion mutant Hae21 was still able to bind to p130. We have previously shown that EBNA-2 is able to form high molecular mass complexes sedimenting at 13S and 34S (Grässer et al., 1991). Our previous studies suggest that the 34S complex may result in part from oligomerization of EBNA-2 and these findings further suggest that two domains of EBNA-2, one between amino acids 122 and 232 and another between amino acids 232 and 344, are important for self-association (Tsui & Schubach, 1994). The failure of the N-terminal deletion mutant H24AH37 to bind to p130 could be due to the deletion of an epitope that mediates the association with p130. Alternatively, the deleted region of H24AH37 could be essential for the oligomerization of EBNA-2, which in turn might only be able to bind to p130 as an oligomer.

Our finding that EBNA-2 expressed in insect cells is able to interact with histone H1 in vitro is consistent with the observation that EBNA-2 can be found in the chromatin fraction (Grässer et al., 1991). Meanwhile, Tong et al. (1994) have confirmed the association of EBNA-2 with histone H1 in vitro and furthermore showed that a fusion protein between glutathione S-transferase and amino acids 335 to 360 from EBNA-2 is able to bind to histone H1 in far-Western blot analysis. These and other data suggest that the interaction between EBNA-2 and histone H1 is mediated via the basic Arg-Gly domain of EBNA-2. We also tested our EBNA-2 mutants for their ability to associate with histone H1 in far-Western blotting (Fig. 2a, b). All mutants tested were able to bind to histone H1 and no significant differences in the amount of bound EBNA-2 were observed. This was surprising in that the mutants ΔNcoKT3 and H28ΔA63 are completely deleted in the Arg-Gly region but still bound to histone H1 (Fig. 2a, b). We therefore hypothesize that the interaction of EBNA-2 with histone H1 might be mediated via more than one domain, thus explaining why these two mutants were still able to bind H1.

In order to investigate which regions of EBNA-2 are essential for indirect binding to DNA, all the mutants baculoviruses expressing the mutant EBNA-2 proteins were generated by cotransfection of Spodoptera frugiperda Sf158 cells with the respective recombinant baculovirus transfer vectors (pACYM1 was used for all plasmid constructions) and linearized BaculoGold virus DNA (Pharmingen) using lipofection as described (see King & Possee, 1992 for details and references). MAb R3 (rat IgG2a; Kremmer et al., 1995) and 6C8 (rat IgG2a) were used for detection of the EBNA-2 proteins. MAb 6C8 (rat IgG2a) was raised against peptide DDDSGPPWPPISD (amino acids underlined correspond to EBNA-2 amino acids 317–327 of EBV strain M-ABA; Cyst198 is replaced by serine) in Lou/C rats exactly as described (Kremmer et al., 1995). bac-wt, Extract of insect cells infected with wild-type baculovirus as negative control; E3-wt, wild-type EBNA-2. The positions of molecular mass marker proteins (Pharmacia) are indicated.
were transcribed-translated \textit{in vitro} using plasmid pSPT19 (Boehringer Mannheim) as the \textit{in vitro} transcription vector. The respective recombinant pSPT19 plasmids were digested with \textit{SalI} and transcribed using SP6 RNA polymerase and a Boehringer Mannheim \textit{in vitro} transcription kit. The resultant RNA was translated \textit{in vitro} using a Promega reticulocyte lysate translation kit. By Western blot analysis we confirmed that all mutants showed the expected migration behaviour and were expressed at similar levels (data not shown). The ability of the various mutants to indirectly bind to a 54 bp TP1 promoter fragment was tested by EMSA as described (Sauder \textit{et al.}, 1994). We observed that the cysteine mutants bound to the TP1 promoter with equal efficiency as the wild-type protein and the linker insertion/deletion mutant KT3 (data not shown). Of the remaining deletion mutants, only the N-terminal mutant Hc24AH37 yielded a signal in the EMSA (Fig. 3a; data not shown). As expected from previously published experiments, no binding of the linker insertion mutant Hae21 was detected (data not shown). The failure of the deletion mutants H20AH28, ΔSph, H37AH21, ΔNco and ΔNcoKT3 and of the insertion mutant Hae21 to bind to the DNA is consistent with the idea that EBNA-2 interacts with RBPJ$\text{\textsubscript{c}}$ via the WWP motif (Trp-Trp$\text{\textsubscript{c}}$-Pro), which is deleted or modified in all of these mutants. In a competition experiment using a peptide containing this motif, Ling & Hayward (1995) recently showed that the interaction between full-length EBNA-2 and RBPJ$\text{\textsubscript{c}}$ could be prevented in an EMSA, implying that EBNA-2 mediates interaction with RBPJ$\text{\textsubscript{c}}$ directly via the WWP motif. Assuming this, MAb 6C8, which was raised against the WWP motif (Fig. 1b), should be able to prevent the association between EBNA-2 and RBPJ$\text{\textsubscript{c}}$. In the experiment presented in Fig. 3 (a) we show that addition of MAb 6C8 to the complex between EBNA-2 and RBPJ$\text{\textsubscript{c}}$ resulted in a dissociation of this complex because no more shift or supershift could be detected in the autoradiograph (lanes 5 and 11). In lane 8, \textit{in vitro}-translated EBNA-2 was incubated with MAb 6C8 prior to addition of EBNA-2-negative nuclear extract, which also led to the same effect as shown in lanes 5 and 11. This experiment provides further evidence for a direct interaction of EBNA-2 with RBPJ$\text{\textsubscript{c}}$ via the WWP motif. By addition of the peptide containing the WWP motif used for the generation of MAb 6C8 to the complex of EBNA-2/RBPJ$\text{\textsubscript{c}}$ bound to the target DNA, we also observed that EBNA-2 was released by this peptide from the DNA as previously shown by Ling & Hayward (1995) (data not shown). The C-terminal mutant H28AΔ63 did not bind to the DNA even though the WWP motif was not deleted. This finding is in good agreement with recently published data by Ling & Hayward (1995), which suggest that regions adjacent to
Mutational analysis of EBNA-2

(a) BL41-P3HR1  Nucleolar extract
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(b) BL41-P3HR1  IB4  Nucleolar extract
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Fig. 3. Analysis of the ability of the EBNA-2 mutant H24AH37 to bind indirectly to a 54 bp TP1 promoter oligonucleotide (a) and interruption of the interaction of EBNA-2 with RBPJκ using a MAb (b). Gel shift reactions were carried out with nuclear extracts of the EBNA-2-negative cell line BL41-P3HR1 or the EBNA-2-positive cell line IB4. Three μl of in vitro-translated protein (i.v.t. protein) containing wild-type EBNA-2 (E2-wt), EBNA2-H24AH37 or in vitro-translated brome mosaic virus RNA (BM) and 3 μl supernatant of either MAb R3 (see Fig. 1b), MAb 6C8 (see Fig. 1b) or anti-mouse CD45 (IgG2a) MAb (as a control) were added as indicated. Complexes I and III represent binding of RBPJκ to one (I) and two (III) binding sites in the 54 bp TP1 oligonucleotide (see Zimber-Strobl et al., 1994). Complex II represents binding of the mutant H24AH37 to RBPJκ, bound to one binding site only because the hypothetical complex consisting of the mutant EBNA-2 plus RBPJκ bound to both sites, expected to migrate between complexes III and IV, was not observed. II* indicates complex II supershifted with MAb R3; IV, complex formed in the presence of in vitro-translated wild-type EBNA-2 or virus EBNA-2 (see Zimber-Strobl et al., 1994); IV*, complex supershifted with MAb R3; lanes 8–10 (b), in vitro-translated EBNA-2 was first incubated with the respective antibodies and then mixed with the remaining components.

the WWP motif are necessary to ensure a stable interaction between EBNA-2 and RBPJκ, at least under the conditions of an EMSA. As mentioned, the cysteine mutants were still able to form complexes with the TP1 promoter in the EMSA.

A study to test the ability of these mutants to transactivate transcription of a TP1–luciferase reporter gene construct in cotransfection assays using the EBNA-2-negative cell line BL41-P3HR1 revealed that mutation of the cysteines did not lead to an abrogation of the ability to transactivate. This suggests that the cysteine residues of EBNA-2 do not play a crucial role in the function of the protein, at least in terms of DNA-binding and transactivation of the TP1 promoter (C. Zabel, C. Sauder, U. Zimber-Strobl, N. Mueller-Lantzsch & F. A. Grässer, unpublished results).

The results from far-Western blotting and DNA-binding analyses are summarized in Fig. 1 (a). The mutants H37AH21, H28AA63, ΔNeoKT3 and Hac21, which did not indirectly bind to the DNA in the EMSA, were still able to associate with the 130 kDa protein, whereas the N-terminal mutant H24AH37, which
retained the ability to bind indirectly to the DNA, showed a markedly reduced association with the 130 kDa protein. Based on these data, we therefore exclude the involvement of the 130 kDa protein in DNA binding, while it is clear that the 63 kDa protein RBPJ\(_{\kappa}\) mediates the interaction between EBNA-2 and its DNA response elements. The mutant T1EBNA-2\(_D\)A19-110 was previously shown by Cohen et al. (1991) to be inactive in both transactivation and transformation assays. Because the N-terminal region deleted in this mutant is covered by the mutant H24AH37 used in our study and because this region is necessary for the interaction between EBNA-2 and the 130 kDa protein, our data suggest that the 130 kDa protein may play a role in the EBNA-2-mediated process of transformation. Studies using the yeast two hybrid system are underway to identify this protein.

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References


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