Characterization of DP103, a Novel DEAD Box Protein That Binds to the Epstein-Barr Virus Nuclear Proteins EBNA2 and EBNA3C*

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The Epstein-Barr virus-encoded nuclear antigens EBNA2 and EBNA3C both interact with the cellular transcription factor RBP-Jκ and modulate the expression of several shared target genes, suggesting a tight cooperation in latently infected cells. In a survey for additional cellular factors that bind to EBNA2 as well as EBNA3C, we have isolated and characterized DP103, a novel human member of the DEAD box family of putative ATP-dependent RNA helicases. The interaction with DP103 is mediated by amino acids (aa) 121–213 of EBNA2 and aa 534–778 of EBNA3C, regions that are not involved in binding of the viral proteins to RBP-Jκ. The DP103-cDNA encodes a protein of 824 aa that harbors all of the common DEAD box motifs. Monoclonal antibodies raised against DP103 detect a protein of 103 kDa in mammalian cells that resides in high molecular weight complexes in vivo. We have detected an ATPase activity intrinsic to or closely associated with DP103. By subcellular fractionation, we find DP103 in both a soluble nuclear fraction as well as in the insoluble skeletal fraction. Whereas the protein and its mRNA are uniformly expressed in all tested cell lines, we observed differential expression of the mRNA in normal human tissues.

The Epstein-Barr virus (EBV), a ubiquitous human γ-herpesvirus, is the etiological agent of infectious mononucleosis and is associated with a number of tumors, such as the endemic form of Burkitt’s lymphoma (BL), nasopharyngeal carcinoma, and Hodgkin’s disease (reviewed in Ref. 1). Infection of primary B cells with EBV in vitro results in continuous proliferation of latently infected B cells, giving rise to lymphoblastoid cell lines (LCLs). Among the restricted set of 9 viral proteins expressed in LCLs are the two nuclear antigens EBNA2 and EBNA3C, both of which are absolutely essential for the ability of EBV to transform B cells in vitro.

EBNA2 is a strong transactivator of the latent viral as well as cellular (CD21, CD23, and c-fer) genes (reviewed in Ref. 1). However, EBNA2 does not bind directly to DNA but targets responsive promoters by binding to the ubiquitously expressed cellular transcription factor RBP-Jκ, a component of the Notch signaling pathway, and the hematopoietic lineage-restricted ets family protein PU.1 (2–8). EBNA3C is also able to interact with RBP-Jκ in vitro and in vivo, resulting in a reduction of RBP-Jκ electrophoretic mobility shift activity and a decrease in the amount of EBNA2/RBP-Jκ complexes (9–12). These observations have led to the suggestion that EBNA3C antagonizes the function of EBNA2 by competing for RBP-Jκ, a model supported by the finding that transient expression of EBNA3C down-regulates the EBNA2-dependent transactivation of the viral LMP1 and LMP2A promoters (9, 13, 14). Other data demonstrate that EBNA3C may also directly modulate transcription from the viral C promoter as well as expression of the viral LMP1 and the cellular CD21 genes independent of EBNA2 (15–18). Taken together, these data suggest that EBNA2 and EBNA3C cooperate in the course of B cell immortalization on multiple levels, including competition for cellular factors as well as direct modulation of the expression of shared target genes. The studies described here were undertaken to identify additional cellular factors that may participate in this complex regulation. We report the cloning and characterization of DP103, a novel member of the DEAD box family of putative ATP-dependent RNA helicases, which was isolated due to its ability to bind to EBNA2 as well as EBNA3C.

The rapidly growing DEAD box family includes members from a broad range of pro- as well as eukaryotic organisms (for a review see Ref. 19). The family’s name is derived from the amino acid sequence Asp-Glu-Ala-Asp (DEAD), one of at least eight highly conserved motifs shared by the family members. The conserved motifs are separated by similar spacings and are arranged in a common core region as represented by the prototype of the family, the DEAD box protein eIF-4A. Based on the observation that this core region harboring the full set of conserved motifs is present in all family members, they are all thought to act as RNA helicases, although helicase activity has been demonstrated for only a minority of DEAD box proteins. In many family members, the core region is flanked by N- or C-terminal extensions sharing little or no sequence homology, suggesting a role for these regions in more specialized functions. DEAD box proteins have been shown to play important roles in cell development, differentiation, and proliferation. They are implicated in nearly all processes that are linked to RNA metabolism, such as translation initiation, pre-mRNA splicing, ribosome assembly, mRNA stabilization, and mRNA transport (reviewed in Refs. 19 and 20).

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF106019.

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† The abbreviations used are: EBV, Epstein-Barr virus; BL, Burkitt’s lymphoma; LCL, lymphoblastoid cell lines; mAbs, monoclonal antibodies; aa, amino acids; PCR, polymerase chain reaction; 5′-RACE, rapid amplification of 5′-cDNA ends; PBS, phosphate-buffered saline; bp, base pair(s); Ni-NTA, nickel-nitrilotriacetic acid; DSP, dithiothreitol; succinimidyl propionate; eIF, eukaryotic initiation factor.

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**Experimental Procedures**

**Cells and Antibodies**—All non-adherent B and T cell lines were maintained in RPMI 1640 medium (Life Technologies, Inc.). Adherent mammalian cell lines were grown in Dulbecco’s modified Eagle medium (Life Technologies, Inc.). The insect cell line SF158 (21) was kept at 27 °C in TC100 medium (Life Technologies, Inc.). All media were supplemented with 10% fetal calf serum (Seromed), 40 IU/ml penicillin, and 50 μg/ml streptomycin. Recombinant baculoviruses His8-PD103 expressing the full-length or DP103ΔN expressing an N-terminal truncated DP103 were generated by lipofection (InsectInus™, Invitrogen) of plasmid pBHHis2B:dp103 with Bac-N-Blue™ (Invitrogen) or pACYC1dp103ΔN with BacuGold® (PharMingen) DNA, respectively, into SF158 cells as described elsewhere (22). Recombinant baculoviruses expressing ENBA2 of the type 1 EBV strain M-ABA were described previously (29). Recombinant baculoviruses expressing His6-tagged full-length ENBA3C with an N-termnally fused His6-tag were a generous gift from Marion Buck and Tom Sculley (Queensland Institute of Medical Research, Brisbane, Australia).

The rat monoclonal antibodies (mAbs) R3 directed against ENBA2 and 1H-4 directed against ENBA1 have been described (23, 24). The mouse mAb A10 directed against ENBA3C (25) was kindly provided by Kenia Krauer (Queensland Institute of Medical Research, Brisbane, Australia). For a review of the yeast two-hybrid system, see Ref. 28. The cDNA library and yeast strains used for the two-hybrid screen with plasmid pBTH116::E2a (encoding an aa 22–344 of the type 1 EBV strain B95-8) have been described previously (29). The library clones isolated from this screen, expressing the cDNA inserts as fusion proteins with the transactivation domain of GAL4 (GAL4AD), were co-transformed into the yeast strain SFY526 (30) as described (31) together with various deletion mutants of ENBA2A, -B, and -C3 cloned in the vector pGBT9. Co-transformants were grown on 100-mm plates with synthetic medium lacking tryptophan and uracil at 30 °C until colonies were 2 mm in diameter. The β-galactosidase activity was determined by the hydrolysis of 5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside (X-Gal) in situ (32).

**Rapid Amplification of 5′-cDNA Ends (5′-RACE)—The lacking 5′-region of the DP103 encoding cDNA was isolated by performing three successive rounds of 5′-RACE. Briefly, mRNA from 8 × 10⁷ BJAB cells was prepared using the mRNA Isolation Kit (CLONTECH) for the production of rat mAbs directed against different epitopes of DP103, either amino acids 713–824 or 352–614 of DP103 were expressed in rat mAbs directed against EBNA2 or EBNA3C. For the generation of plasmid pATH2:dp103CT or pATH2:dp103ΔN, a segment encoding amino acids 713–824 from the open region of the Superscript™ reverse transcriptase (Life Technologies, Inc.). The reaction was primed with Superscript™ reverse transcriptase (Life Technologies, Inc.). The reaction was primed with DP103 cDNA-specific primers R01 (5′-TACC-AATATCCCTGGGCTGGTCAGG), R03 (5′-CAACAGTATGCA-GTGCAGCCAAC), or R05 (5′-AAAGCTTGAATATGGAAATT-GCTG) in the first, second, or third round of RACE, respectively. The reaction was carried out either at 42 °C or, in order to overcome secondary structures, at 51 °C for 30 min. Subsequent steps were performed using the Marathon™ cDNA Amplification Kit (CLONTECH—Eching according to the manufacturer’s instructions. Briefly, double-stranded cDNA was generated, ligated to an adaptor (CLONTECH), and subjected to PCR amplification with an 5′-primer specific for the adaptor and DP103-specific primers R02 (5′-TGTCTTATCTGGTCTG-CCATG), R04 (5′-CGGATCCCTTCTCCGACATACG), or R06 (5′-CCCTCCAAAGACATGACACTTAAACCC) in the first, second, or third round of RACE, respectively. Products were subsequently cloned in the vector pGEM (Promega).

**Plasmid Constructs**—For the generation of plasmid pATH2: dp103CT, a segment encoding amino acids 713–824 from the open reading frame of the DP103-cDNA was amplified by polymerase chain reaction (PCR) using primers DP103Ba-mHII(352) (5′-GCAGUGGCTGATGATCTGGA) and DP103Sa-mHII(614) (5′-GCCTGTTGCAATCCGATTTCC). Plasmid pATH2: dp103ΔN was constructed by digestion of the first RACE product with BamHI and PflMI and ligation to the BamHI/ PflMI-cleaved pAT-H2:dp103CT vector. Removal of the insert with BamHI and BglII and cloning into the BamHI site of pACYC1 yielded plasmid pACYCl-dp103ΔN. The recombinant protein was expressed as a non-fusion protein initiating with the ATG codon at position 1064, comprising amino acids 352–614 of DP103. Plasmid pGEM-dp103 was obtained by reverse transcriptase-PCR amplification of the complete coding region of the DP103-cDNA with primers DP103I/5′ (CCATGGCCGGGGA- GTGGAAG) and DP103I/2631/3′ (ATGATGAT GTCCGTTCAATGGAAGA ACTC) and subsequent ligation to the linearized vector pGEM (Promega).

For in vitro translation, the insert from pGEM-dp103 was removed using the NcoI and SalI-cleaved pUC19 vector to yield plasmid pGEM-dp103I. Plasmid pBHHis2B:dp103 was generated by removal of the insert from pGEM-dp103I by NcoI/Sall cleavage (vector Invitrogen). This construct expresses DP103 as a fusion protein with an N-terminal tag consisting of an antibody epitope (Anti-Xpress, Invitrogen) and a His6-tag. The GAL4 DNA binding domain (GAL4BD) fusions of ENBA2 and ENBA3C were constructed in the vector pGBT9 (30). For cloning of pGTB9-E2a (1–1,213), the corresponding region of ENBA2 from the EBV type 1 strain M-ABA was PCR-amplified with primers E2AecoRI(1′/5′, GCCGACTGCATGCTACTTCTATTTGCG) and E2Aso-RI(213′/5′, GTCGACTGTGGACCGCTTAGTTGACGGAAG) digested with EcoRI and Sall, and subsequently cloned into the EcoRI/Sall-cleaved pGBT9. Additional fusions of ENBA2 and ENBA3C were generated in a similar way using primers with suitable restriction sites. Sequence information concerning these primers can be obtained from the authors upon request. Two pGBT9 constructs expressing GAL4BD fusions of an 127–985 of ENBA3C from the type 1 EBV strain B95-8 and aa 127–1062 of ENBA3C from type 2 EBV strain AG576 were kindly provided by Kenia Krauer (Queensland Institute of Medical Research, Brisbane, Australia).

**Zone Velocity Sedimentation—Soluble extracts of 2 × 10⁷ EBV-positive and EBV-negative cells were separated by centrifugation through a 5–30% sucrose gradient as described (27). Fifteen fractions collected by bottom puncture were precipitated with ethanol and analyzed by immunoblotting.

**Northern Blotting—**Total cellular RNA from cultured cells was isolated using the “RNAeasy kit” (Qiagen), following the manufacturer’s instructions. Isolation of total RNA from tissues was done as described (33). For the generation of probes, nucleotides 1654–2113 of the DP103-cDNA from plasmid pATH2:dp103ΔN were removed by digestion with EcoRI and KM. Multiprime labeling of the probe, blot transfer, and hybridization were performed as described (35).

**Cell Surface Immunocytochemistry—**B cells were approximately 1 × 10⁷ SF128 cells were infected with recombinant baculoviruses expressing full-length His6-tagged ENBA3C, full-length ENBA2, or N-terminally truncated DP103ΔN protein as described previously (22). Cells were washed twice in ice-cold PBS and lysed in 500 μl of ice-cold lysis buffer NBL (150 mM Tris-Cl, pH 9.0, 150 mM NaCl, 1 mM β-mercaptoethanol, 10% glycerol, 0.5% Nonidet P-40) supplemented with a protease inhib-
samples were incubated again for 1 h. Pellets were washed five times with lysis buffer and boiled in SDS-sample buffer containing β-mercaptoethanol for 5 min. Samples were analyzed by immunoblotting.

**ATPase Assays**—2 × 10^6^ cells from suspension cultures were pelleted, washed twice in ice-cold PBS, and lysed for 30 min on ice in 1 ml of buffer K (see above) with 1 mM DTT. After centrifugation (13,000 × g, 30 min, 4 °C), the clarified supernatants were pre-adsorbed on 150 μl of a 1:1 slurry of protein A-Sepharose beads (Amersham Pharmacia Biotech) in PBS at 4 °C for 2 h on a rotating wheel. The DP103-specific mAb 9A-3 or an irrelevant mAb directed against TrpE were added to the supernatants at a final concentration of 25 ng/μl. The samples were incubated at 4 °C overnight, and 50 μl of a 1:1 slurry of protein A-Sepharose beads in PBS were added. After incubation at 4 °C on a rotating wheel for 1.5 h, the beads were washed three times in ice-cold lysis buffer, once in ice-cold ATPase buffer (20 mM HEPES, pH 7.2, 100 mM NaCl, 5 mM MgCl2), and twice in ice-cold ATPase buffer supplemented with ATP (Roche Molecular Biochemicals) at a final concentration of 50 μM. The samples were supplemented with 800 ng of total cellular RNA isolated from B95-8 or Akata cells and brought to a final volume of 80 μl with ice-cold ATPase buffer. After addition of 1 μCi of [α-32P]ATP (ICN), reactions were incubated at 37 °C. After 0.5, 1, 1.5, and 3.5 h of incubation, 1 μl of the samples were spotted on TLC plates (Sigma). TLC plates were developed in 0.75 M KH2PO4, pH 3.0, and subjected to autoradiography.

**Sequence Analysis**—Data base searches were performed using the BLOCKS, BLASTP, and TBLASTN programs (35, 36). Hydropathy prediction by DP103 was determined according to the method of Kyte and Doolittle (37) over a window length of 17 residues. All programs were accessed through the Baylor College of Medicine search launcher internet pages.

**RESULTS**

**Isolation of a cDNA Clone Interacting with EBNA2 and EBNA3C**—In a search for proteins that interact with both EBNA2 and EBNA3C, we determined whether candidate clones identified in a previous two-hybrid screen for EBNA2-associated proteins could also bind to EBNA3C in this assay. Details of the original screen have been described previously (29). The GAL4AD-fusion protein encoded by the cDNA clone SE97 induced β-galactosidase activity when co-expressed with the full-length EBNA3C fused to the DNA binding domain of GAL4. To map further the regions mediating the interaction, additional deletion mutants of EBNA2 and EBNA3C from the type 1 EBV strains M-ABA or B95-8, respectively, were co-expressed with SE97. As shown in Fig. 1, the smallest fragments that conferred binding to SE97 comprised an 121–213 of EBNA2 and an 534–778 of EBNA3C. Since these regions are poorly conserved between the EBNA2 and EBNA3C alleles of the type 1 and type 2 viruses, additional mutants of EBNA2 and EBNA3C from the type 2 strains Jijoye or AG876, respectively, were employed in the yeast two-hybrid system. In both cases, the EBV type 2-derived mutants induced β-galactosidase activity when co-expressed with SE97 (Fig. 1). Thus, the ability to interact with the protein encoded by SE97 is conserved between the EBNA2 and EBNA3C alleles of both types 1 and 2 EBV strains.

**SE97 Encodes a C-Terminal Fragment of DP103, a Novel Member of the DEAD Box Family**—The cDNA insert in SE97 comprised 793 bp with an open reading frame of 479 bp. The clone was assumed to be incomplete since it lacked both a candidate start codon and a polyadenylation signal. Furthermore, this sequence hybridized to a unique transcript of approximately 3.0 kilobase pairs in Northern blots (see Fig. 7A). Using mRNA derived from the EBV-negative B cell line BJAB, a segment containing an additional 2003 bp was obtained by 5′-RACE. The complete 3′-end of the clone is likely to be represented by an expressed sequence tag (EST) of 140 bp derived from human tonsil cells (GenBank™ accession number AA731204). The EST shows a perfect match of its 5′-terminal 74 bp to the 3′-end of the library clone and harbors a polyadenylation signal (AAUAAA) at position 101, 18 bp upstream of the binding site of the oligo(dT) primer. The size of the cloned sequence together with the 3′-region covered by the EST is 2842 bp and correlates with the size of the transcript observed in Northern blotting, assuming an additional 200 bases for an average sized poly(A)-tail (38). Moreover, two additional independent 5′-RACE experiments yielded products terminating at the same 5′-position. Thus, the cloned sequence is likely to represent the complete 5′-end of the DP103-encoding transcript.

To rule out the possibility that the sequence retrieved by RACE resulted from nonspecific cDNA fusion or was unique to the starting material used, cDNAs spanning the entire 2796 bp were amplified, cloned, and sequenced, using mRNA derived from human placenta, the human B cell line BJAB, as well as...
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Fig. 2. Predicted amino acid sequence of DP103. The cDNA sequence of the first 19 nucleotides and the 3’-untranslated region including the stop codon are shown above and under the amino acid sequence, respectively. The putative start codon and the Kozak consensus sequence (39) are underlined. Conserved amino acid residues of the DEAD box family (19) are shown boxed. The basic segment spanning aa 720–741 as well as the repetition of the first 5 aa of this segment (KTRLK) at position 55–59 are shown in bold.

The DP103 Gene Encodes a Phosphoprotein of 103 kDa—As predicted from the amino acid composition of the protein encoded by DP103, in vitro transcription/translation yielded a protein with an apparent mass of about 92.5 kDa (Fig. 3A, lane 6). For further biochemical analysis, rat monoclonal antibodies detect a cellular protein with an apparent molecular mass of 103 kDa (Fig. 3A, lane 7). Since the molecular mass calculated for the 41-aa tag of the recombinant His6DP103 protein is 4.6 kDa, we expected to observe an endogenous protein of approximately 105 kDa in extracts of mammalian cells. Indeed, a protein with an apparent molecular mass of 103 kDa was detected by both mAb 9A-3 and the rabbit serum 2361, designated His6-DP103, respectively (Fig. 3A). The rabbit serum raised against the recombinant DP103 in vitro-translated protein also reacted with the 103-kDa protein in Western blotting but exhibited background staining of additional proteins (data not shown).

Infected with DP103ΔN or His6-DP103, respectively (Fig. 3A). Since the molecular mass calculated for the 41-aa tag of the recombinant His6-DP103 protein is 4.6 kDa, we expected to observe an endogenous protein of approximately 105 kDa in extracts of mammalian cells. Indeed, a protein with an apparent molecular mass of 103 kDa was detected by both mAb 9A-3 and the rabbit serum 2361, designated His6-DP103, respectively (Fig. 3A). The rabbit serum raised against the recombinant DP103 in vitro-translated protein also reacted with the 103-kDa protein in Western blotting but exhibited background staining of additional proteins (data not shown).

The assumed association of DP103 with the nuclear antigens EBNA2 and EBNA3C predicts that it should be present in the nucleus of the cell. We performed subcellular fractionation of EBV-positive and EBV-negative B cell lines as described previously (27, 40). Soluble fractions from the nucleus and cytoplasm were analyzed by immunoblot as shown in Fig. 3B. The 103-kDa protein was observed in EBV-positive and EBV-negative B cell lines as well as in the T-cell line Jurkat, in SK-N-S-H neuroblastoma cells and HeLa cells (band designated a in Fig. 3B). In addition to the major immunoreactive band at 103 kDa, we also observed binding to a protein migrating with an apparent molecular mass of 130 kDa (band designated b in Fig. 3B). This band was present in all cell lines tested but was most pronounced in the B95-8 and M-ABA cell lines. Since the mAb 8H-4 reacted only with p103, we assume that the recognition of the 130-kDa protein is due to a cross-reactivity of the mAb 9A-3. The rabbit serum raised against the TrpE fusion protein also reacted with the 103-kDa protein in Western blotting but exhibited background staining of additional proteins (data not shown).

The assumed association of DP103 with the nuclear antigens EBNA2 and EBNA3C predicts that it should be present in the nucleus of the cell. We performed subcellular fractionation of EBV-positive and EBV-negative B cell lines as described previously (27, 40). Soluble fractions from the nucleus and cytoplasm were analyzed by immunoblot as shown in Fig. 3C. These results showed that DP103 was almost exclusively detected in the soluble nuclear fractions of all cell lines tested. This result was confirmed by detection of EBNA2 in the nuclear fractions of the cell lines B95-8 and Raji using the mAb R3 (23). As expected, EBNA2 was absent from the EBV-negative cell line BJAB. To separate further the membrane and skeletal fractions, extracts were prepared following the method described in Ref. 41. By this method, the cell lysate is separated into a soluble nuclear fraction that includes chromatin-bound proteins, a soluble cytoplasmic fraction, a fraction containing membrane structures, and a fraction containing the nuclear membrane and cytoplasmic proteins (data not shown).
matrix and the cytoskeleton. The analysis of the presence of both DP103 and EBNA2A in such fractionated cell extracts from Raji (EBNA2-positive) and BJAB cells (EBV-negative) is shown in the lower panel of Fig. 3C. In both cell extracts, DP103 was again readily detected in the soluble nuclear fraction (N) and at low levels in the cytosol (C) and membranous fraction (M) that is likely due to cross-contamination. In addition, about half of the DP103 protein was found in the insoluble part (S), representing the nuclear matrix and the cytoskeleton. The presence of EBNA2A in the soluble nuclear as well as the insoluble fraction again confirms earlier data showing this subcellular distribution of EBNA2 (27, 42).

To determine whether DP103 is phosphorylated in vivo, immunoprecipitation experiments were carried out using extracts of Raji cells labeled with either [35S]methionine or [32P]P, (Fig. 4), using either a polyclonal rabbit serum (lanes designated ser2361), non-immune serum of the same animal (lanes designated pre2361), the mAb 9A-3 (lanes designated 9A-3), or an irrelevant mAb directed against bacterial TrpE protein (lane designated aTrpE). The irrelevant aTrpE control shown on the right of Fig. 4 was applied together with the same immunoprecipitates from [35S]methionine-labeled cells using mAb 9A-3 as shown on the left on a higher percentage gel. Both the rabbit immune serum and the mAb 9A-3, but not the negative control antibodies, precipitated a protein migrating at approximately 103 kDa from [35S]-labeled cell extracts (indicated by arrows in Fig. 4), thus confirming the results obtained in the Western blot analysis. Also, both 9A-3 and the immune serum precipitated a phosphoprotein with a mobility of 103 kDa from [32P]-labeled cell extracts which was undetectable using the preimmune serum (lanes designated “pre2361”) and the irrelevant mAb (data not shown). The phosphoprotein of 130 kDa detected exclusively by mAb 9A-3 presumably is identical to the 130-kDa protein that the antibody detects in immunoblots (Fig. 3A). Additional proteins were precipitated along with the 103-kDa protein from [32P]-labeled cell extracts, which were also detectable in [35S]-labeled cell extracts. Most prominent are phosphoproteins of approximately 160 kDa precipitated by the mAb 9A-3 and of 135 and 60 kDa precipitated by the serum. A phosphoprotein of 40 kDa was precipitated by both antibodies.

To investigate further the possibility that DP103 is part of a heteromeric protein complex, we performed zone velocity sedimentation with soluble, native extracts prepared from the cell lines BL41, Raji, and B95-8 loaded on 5–30% sucrose gradients. As shown in Fig. 5, DP103 was exclusively detected in fractions of high molecular weight (fractions 1–6) with a peak in fraction 1, whereas none or only very low amounts of the protein were present in low density fractions (Fig. 5, A–C). In addition, the 130-kDa protein detected by the mAb 9A-3 was observed in the fraction expected for the monomeric form of the protein (fraction 11 in Fig. 5C) and thus seems not to be a component of the...
DP103-containing complexes. The migration of EBNA2 in the extracts from Raji and B95-8 cells is shown in Fig. 5, D and E, respectively. As described previously (27), about 20% of EBNA2 was found in the high density fractions with a peak in fraction 1. We point out that the co-sedimentation of EBNA2 and DP103 in the fractions of very high molecular weight supports but does not prove the notion that the EBNA2 and DP103 interact in EBNA2-positive cell lines.

In summary, our results demonstrated that DP103 encodes a nuclear phosphoprotein with an apparent molecular mass of approximately 103 kDa, which almost exclusively resides in high molecular weight complexes in vivo.

**EBNA2 and EBNA3C Bind to DP103 in Vitro**—To confirm the interaction of both EBNA2 and EBNA3C with DP103 by a second method, in vitro binding studies were carried out employing EBNA2A, EBNA3C, and DP103 expressed in insect cells. Initially, we intended to use His$_6$-tagged DP103 bound to nickel-agarose for affinity chromatography. However, pilot experiments revealed that EBNA2 bound efficiently to nickel-agarose and could even be released from the resin using an imidazole gradient. Since the 14 histidine residues that are present within the EBNA2 linear amino acid sequence do not form any stretches of more than two contiguous histidines, we conclude that several of these histidines reside on the surface of the protein, thus allowing efficient chelating of Ni$_2^+$ ions. Therefore, we chose an experimental procedure in which resins loaded with either EBNA2 or EBNA3C were incubated with extracts from insect cells infected with either recombinant baculovirus expressing EBNA2A (A), His$_6$-tagged EBNA3C (B), or wild-type baculovirus (C). The input is shown in the lanes designated C(1), the following lanes FT, W1, and W20 show the flow-through as well as the first and last of 20 wash fractions. Lanes designated C(2) and following lanes FT, W1, and W20 show the input, flow-through, and wash fractions of the subsequent incubation of the resin with DP103. EBNA2 and EBNA3C were detected with mAbs R3, A10, and 9A-3, respectively. Upper blots shown in A and B were probed first with mAb R3 and A10, respectively, and subsequently with mAb 9A-3 (lower blots) without prior stripping. D, co-immunoprecipitation of cross-linked EBNA2A/DP103. EBV-positive B95-8 or EBV-negative BJAB cells were treated with DSP (Lomant’s reagent), and cell extracts were subjected to immunoprecipitation using the antibodies 9A-3 or 8H-4 directed against DP103, R3 directed against EBNA2, respectively. The lanes designated control show the input prior to immunoprecipitation. The Western blots were either stained stained with the DP013-specific antibody 8H-4 (top panel) or with the EBNA-2-specific antibody R3 (lower panel). The small arrow indicates the position of EBNA2 co-precipitated by the DP103-specific antibody 9A-3.

**FIG. 6.** A–C, affinity chromatography of DP103 on Ni-NTA-agarose loaded with with EBNA2 or EBNA3C. Ni-NTA resins were incubated with extracts from insect cells infected with either recombinant baculovirus expressing EBNA2A (A), His$_6$-tagged EBNA3C (B), or wild-type baculovirus (C). The input is shown in the lanes designated C(1), the following lanes FT, W1, and W20 show the flow-through as well as the first and last of 20 wash fractions. Lanes designated C(2) and following lanes FT, W1, and W20 show the input, flow-through, and wash fractions of the subsequent incubation of the resin with DP103N expressed in insect cells. Bound proteins were eluted with an imidazole step gradient as indicated. EBNA2A, EBNA3C, and DP103N were detected with mAbs R3, A10, and 9A-3, respectively. Upper blots shown in A and B were probed first with mAb R3 and A10, respectively, and subsequently with mAb 9A-3 (lower blots) without prior stripping. D, co-immunoprecipitation of cross-linked EBNA2A/DP103. EBV-positive B95-8 or EBV-negative BJAB cells were treated with DSP (Lomant’s reagent), and cell extracts were subjected to immunoprecipitation using the antibodies 9A-3 or 8H-4 directed against DP103, R3 directed against EBNA2, respectively. The lanes designated control show the input prior to immunoprecipitation. The Western blots were either stained stained with the DP013-specific antibody 8H-4 (top panel) or with the EBNA-2-specific antibody R3 (lower panel). The small arrow indicates the position of EBNA2 co-precipitated by the DP103-specific antibody 9A-3.
tagged p40 protein of Borna disease virus\(^3\) did not retain DP103\(\Delta N\) (data not shown). In contrast, we could clearly detect co-elution of DP103\(\Delta N\) with EBNA2A or EBNA3C from appropriately loaded resins (Fig. 6, A and B). These experiments demonstrated that both EBNA2A and EBNA3C are able to interact in vitro with truncated DP103 and thus confirm our results obtained in yeast cells.

Experiments to co-precipitate the proteins from native cell extracts were largely negative, probably because the interaction is transient or involves only sub-fractions of the proteins (see "Discussion"). Therefore, we subjected EBV-negative BJAB and EBV-positive B95-8 cells to chemical cross-linking in vivo using DSP (Lomant's reagent), a membrane-permeable bifunctional linker with an internal disulfide bond that can be cleaved prior to SDS-gel electrophoresis by use of a buffer containing \(\beta\)-mercaptoethanol. The results of such an experiment are shown in Fig. 6D. We could demonstrate co-precipitation of EBNA2 and DP103 using mAb 9A-3 from DSP-treated B95-8 cells. With the DP103-specific mAb 8H-4, we also obtained a very faint signal of co-precipitated EBNA2, which was, however, too weak to be photographically reproduced. In the negative control samples employing the EBNA1-specific antibody IH-4, no DP103 or EBNA2 was detected. Likewise, no EBNA1 protein was co-precipitated along with DP103 by mAbs 9A-3 and 8H-4 (data not shown). We were, however, not able to detect a co-precipitation of DP103 and EBNA3C. The detection of EBNA2 in immunoprecipitates of the mAb A10 again confirms earlier data reporting precipitation of EBNA2 by this EBNA3C-specific mAb (25). The corresponding experiments carried out with EBV-negative cell extract showed that the DP103 protein was precipitated; as expected, no EBNA2 or EBNA3C was detectable.

Expression of DP103 in Cell Lines and Tissues—Initial Northern blot experiments using the SE97 sequence as a probe revealed a band with a mobility of approximately 3000 bases using RNA isolated from HeLa cells, primary lymphocytes, or human placenta. The expression of DP103 was also tested in various cell lines and tissues where we observed a band with the same mobility. A representative Northern blot employing various EBV-negative and EBV-positive B cell lines is shown in Fig. 7A. We found comparable levels of expression in all of these cell lines. In contrast, we observed that DP103 was differentially expressed in various primary human tissues as shown in Fig. 7B. Expression was most pronounced in testes and tonsils. After 10 days of exposure to the autoradiogram, expression of DP103 was detectable also in colon, skeletal muscle, liver, kidney, and lung, indicating a lower level of expression in these tissues. No signals were detectable in brain, prostate, stomach, and primary peripheral blood lymphocytes. Interestingly, we observed strong expression of the full-length mRNA in primary human malignant melanoma tumor samples (lanes MM1, MM2, and MM3).

ATPase Activity of DP103 Precipitated from B Cells—In order to test endogenously expressed DP103 for enzymatic activity, ATPase assays were performed with the cellular protein precipitated by the mAb 9A-3 from the B cell lines B95-8 (EBV-positive LCL) and Akata (EBV-positive BL cell line). The cell line Akata expressed DP103 to similar levels as the cell lines depicted in Fig. 3B (data not shown). The precipitated protein bound to protein A-Sepharose beads was incubated with \(\alpha\)-\(^{32}\)P]ATP, and hydrolysis was analyzed by thin layer chromatography. Fig. 8A shows an autoradiogram of aliquots after 1.5 h of incubation. ATPase activity, resulting in the hydrolysis of ATP to AMP, was clearly detectable in the samples containing protein precipitated by the mAb 9A-3 from the LCL B95-8, expressing the latent EBV genes to high levels but was absent from the negative control samples precipitated by the irrelevant mAb αTrpE. In contrast, no detectable ATPase activity was associated with the cellular DP103 protein precipitated from Akata cells, which expressed only very low levels of EBNA2 and EBNA3C (data not shown). To exclude the possibility that the ATPase activity observed in B95-8 cells was due to a cross-precipitated phosphatase, samples taken after different time points up to 3.5 h of incubation were analyzed as shown in Fig. 8B. The AMP was not further hydrolyzed to adenosine and inorganic phosphate, arguing against a contamination by an unspecified phosphatase.

In a different set of experiments, ATPase activity resulting in the hydrolysis of ATP to AMP was also associated with the DP103 protein precipitated from the EBV-positive LCL M-ABA CBL but not with the protein precipitated from the EBV-negative BL cell line BJAB (data not shown).

**DISCUSSION**

We have isolated a cDNA clone encoding a novel DEAD box protein of 824 aa that was initially identified due to its ability to interact with the Epstein-Barr virus-encoded EBNA2 and EBNA3C proteins. Since specific monoclonal antibodies detected an endogenously expressed protein with an apparent molecular mass of 103 kDa, we have named the protein DP103 (DEAD box protein of 103 kDa) and the human gene DP103.

\(^3\)C. Sauder, A. Müller and F. A. Grässer, unpublished observations.
Novel DEAD Box Protein DP103

According to data base searches, the region of similarity to other DEAD box proteins forms a block ranging from aa 74 to 410. Within this block, similarity is restricted mainly to the typical motifs of the family. Thus, DP103 does not represent a homologue or close relative of any known DEAD box protein. The N-terminal segment from aa 1 to 73 and the C-terminal region from aa 411 to 824 do not show any significant homology to other proteins within or outside the DEAD box family. Unique N- or C-terminal extensions are found in most DEAD box proteins, suggesting a role of these regions in specialized functions of the individual proteins (19). Whereas the N-terminal 73 aa of DP103 are overall hydrophobic, two segments within the C terminus from aa 470 to 560 and aa 650 to 824 are highly hydrophilic, suggesting that residues from these regions reside on the surface of the protein. Since DP103 was almost exclusively detected in fractions of high molecular weight in zone velocity sedimentation experiments (Fig. 5), we assume that the C-terminal region of DP103 mediates the interaction with other cellular proteins, which may be important cofactors in the function of DP103. Phosphorylation may also play a role in the modulation of the activity of DP103 in vivo.

Northern blotting with mRNA derived from various normal human tissues indicated differential expression of DP103 with a very high level of expression in testis (Fig. 7B). The lack of detectable expression in non-dividing peripheral blood lymphocytes is in sharp contrast to the readily detectable expression of DP103/DP103 in all permanent cell lines analyzed including B and T cells. In this context, the high expression of the DP103 mRNA in tonsil seems also noteworthy, since these tissues were derived from patients with acute tonsillitis, thus being highly infiltrated with activated B and T cells. We also observed that the neuroblastoma-derived cell line SK-NS-H expresses DP103 at high levels, whereas the DP103-mRNA was undetectable in the brain. In addition, each of the three malignant melanomas that were tested in this survey exhibited high expression levels of the DP103-mRNA. All of the above indicate that DP103 may be highly expressed in proliferating cells. Several DEAD box proteins have been described as factors involved in cell growth and division (43–46). In addition, some human DEAD box proteins were isolated from primary tumor material or tumor-derived cell lines as products of overexpressed or translocated genes, suggesting a possible role for DEAD box proteins in the development of human malignancies (47–51). Studies are presently under way to investigate the expression of DP103 in resting and stimulated peripheral lymphocytes as well as in tumor tissues.

Since DEAD box proteins are generally assumed to be ATPases and ATPase activity has been reported for many family members (for examples see Refs. 52–54), we performed ATPase assays that were initially carried out with DP103 expressed in insect cells. However, no ATPase activity significantly above background levels was observed in experiments employing affinity purified His6-DBP103 (data not shown). One possible explanation for this observation is that insect cells may lack cellular cofactors that are essential for the function of DP103. The enzymatic activity of the DEAD box helicase eIF-4A, for instance, is stimulated 20-fold in the presence of eIF-4B (55, 56). Therefore, additional experiments employing the endogenous protein precipitated from human B cells were performed (Fig. 8). The ATPase activity observed in immunoprecipitates from B95-8 cells is likely to be an intrinsic property of DP103. However, since our assays were carried out using cell extracts, we cannot rule out the possibility that the ATPase activity could have resulted from additional proteins precipitated along with DP103. We did not observe ATPase activity above background levels in the Burkitt’s lymphoma cell lines Akata (EBV-positive) and BJAB (EBV-negative) (Fig. 8B and data not shown). In the cell line Akata, although EBV-positive, we could detect only very low expression of EBNA2 and almost no expression of EBNA3C, whereas DP103 was expressed in similar levels as in the cell lines shown in Fig. 3B (data not shown). Thus, it is possible that expression of viral latent proteins, i.e. EBNA2 and/or EBNA3C, stimulates the ATPase activity of DP103. We are currently performing transient transfection assays in order to investigate the possibility of a direct effect of the viral proteins on the enzymatic activity of DP103. Interestingly, Randall et al. (57) detected an ATPase activity associated with EBNA2. According to a silver-stained gel, the enzymatically active fractions contained additional proteins copurified with EBNA2, one of them apparently migrating with the same mobility as DP103. Thus, at least a part of the ATPase activity observed by Randall and colleagues (57) may have resulted from the co-purification of DP103.

The C-terminal region of DP103 interacting with EBNA2 and EBNA3C comprised aa 666–824. We have mapped the binding regions on EBNA2 and EBNA3C in the yeast two-hybrid system to aa 121–213 of EBNA2A and aa 534–778 of EBNA3C (Fig. 1). Although these regions are poorly conserved between the EBNA2/EBNA3C proteins of the types 1 and 2 EBV strains, both alleles are able to bind to DP103, an observation that argues for the specificity and significance of the interaction. However, although the interaction was confirmed by a second method in vitro, we were only able so far to demonstrate binding of EBNA2 to DP103 by co-purification from B cell extracts after subjecting the cells to the chemical crosslinker DSP. There are several possible explanations for this result. The interaction between DP103 and EBNA2/EBNA3C may be transient or only small fractions of the proteins below the level of detection might be complexed in vivo. It was not

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**Fig. 8. ATPase activity of DP103.** Hydrolysis of [α-32P]ATP in immunoprecipitations using the mAb 9A-3 or an irrelevant control antibody directed against TrpE (αTrpE) from extracts of B95-8 or Akata cells was analyzed by TLC and subsequent autoradiography. Positions of ATP, ADP, and AMP are indicated. A, analysis of aliquots of the reaction mixtures developed after 1.5 h of incubation at 37 °C. Cell extract was omitted for the determination of background as shown in the autoradiogram labeled ϕ. B, time course of ATP hydrolysis in samples from B95-8 cells after 0.5, 1, 1.5, and 3.5 h of incubation. Autoradiograms were exposed for 24 h in A or 12 h in B.
possible to overexpress DP103 in mammalian cells using various expression constructs, an observation that raises the possibility that the expression of the protein may be regulated on the post-transcriptional level. Given that DP103 exclusively forms heteromeric high molecular weight complexes, whereas the major parts of EBNA2 and EBNA3C are detectable in fractions of lower molecular weight, only a relatively small fraction of either EBNA2 or EBNA3C might be bound to DP103 at a given time. The interaction between EBNA2/EBNA3C could also require an intact complex that may be refractile to immunoprecipitation. Although we were able to show co-immunoprecipitation of cross-linked EBNA3C/DP103, we were unable to show co-precipitation of cross-linked EBNA2 and DP103, although we were able to show co-immunoprecipitation of cross-linked EBNA2 and DP103. This might represent a technical problem due to the fact that the available monoclonal antibodies directed against EBNA3C produce only weak signals even when EBNA3C is expressed in insect cells, whereas endogenous or recombinant EBNA2 is readily detectable. The interaction between EBNA3C and DP103 was, however, observed independently in the yeast two-hybrid system and by in vitro affinity chromatography. Therefore, we believe that the interaction between the two proteins is not artifactual.

Although the interaction of DP103 with EBNA2 and EBNA3C requires further investigation, it may help to delineate attractive models for the function of DP103. As important mediators of RNA metabolism, DEAD box proteins are interesting targets for viral proteins that modulate gene expression. There is growing evidence that transcriptional and post-transcriptional processes such as pre-mRNA splicing are tightly linked rather than being compartmentalized (Refs. 58–60 and 84).

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REFERENCES
1. Kieff, E. (1996) in Virolology (Fields, B. N., Knipe, D. M., Howley, P. M., Chanock, R. M., Melnick, J. L., Monath, T. P., Roizman, B., and Straus, S. E., eds) pp. 2343–2496, Raven Press, Ltd., New York.
2. Zimber-Strobl, U., Strobl, L., Metinger, C., Hinrichs, R., Sakai, T., Furukawa, T., Honjo, T., and Bornkamm, G. W. (1994) EMBO J. 13, 4973–4982.
3. Henkel, T., Ling, P. D., Hayward, S. D., and Petersen, M. G. (1994) Science 265, 92–95.
4. Ling, P. D., Hsieh, J. J., Ruf, I. K., Rawlins, D. R., and Hayward, S. D. (1994) J. Virol. 68, 5375–5383.
5. Grossman, S. R., Johannsen, E., Tong, X., Yamamoto, K., and Elkon, R. I. (1994) Proc. Natl. Acad. Sci. U.S.A. 91, 1663–1667.
6. Walter, L., Page, S., Sauder, L., and Pozuelos, R. (1994) J. Virol. 68, 4938–4946.
7. Ackroyd, M., MacKenzie, C., and McManus, M. (1994) Nucleic Acids Res. 22, 233–241.
8. Rozen, F., Edery, I., Meerovitch, K., Dever, T. E., Merrick, W. C., and Sonenberg, N. (1990) EMBO J. 9, 3624–3630.
9. Cuny, G., Cuny, M., and Huber, B. (1990) Mol. Cancer Res. 1, 83–90.
10. Ling, P. D., and Hayward, S. D. (1995) J. Virol. 69, 4538–4548.
11. Rosman, D., and Lane, D. P. (1989) EMBO J. 8, 2847–2852.
12. Rozen, F., and Sonenberg, N. (1990) Mol. Biol. Cell. 1, 1134–1144.
13. Hayflick, L., and Moorhead, P. S. (1961) Virology 20, 581–592.
14. Zimber-Strobl, U., and Bornkamm, G. W. (1994) J. Virol. 68, 6894–6898.
15. Zimber-Strobl, U., and Bornkamm, G. W. (1994) J. Virol. 68, 6894–6898.
16. Zimber-Strobl, U., and Bornkamm, G. W. (1994) J. Virol. 68, 6894–6898.
17. Zimber-Strobl, U., and Bornkamm, G. W. (1994) J. Virol. 68, 6894–6898.
18. Zimber-Strobl, U., and Bornkamm, G. W. (1994) J. Virol. 68, 6894–6898.
19. Zimber-Strobl, U., and Bornkamm, G. W. (1994) J. Virol. 68, 6894–6898.
20. Zimber-Strobl, U., and Bornkamm, G. W. (1994) J. Virol. 68, 6894–6898.