

Epstein-Barr Virus Nuclear Antigen 1 Forms a Complex with the Nuclear Transporter Karyopherin $\alpha 2$ *

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The Epstein-Barr virus (EBV) is implicated in the induction of several malignancies. The nuclear antigen 1 (EBNA1) is the only viral protein that is expressed consistently in all EBV-associated tumors. EBNA1 is involved in the replication and maintenance of the viral episome in the infected cell and exhibits oncogenic activity in transgenic mice. Here we report the identification of the nuclear transporter karyopherin $\alpha 2$ as a cellular partner of EBNA1 using the yeast “two-hybrid system.” Karyopherin $\alpha 2$ is also called importin α or Rch1. The binding to karyopherin $\alpha 2$ was mediated through a C-terminal region of EBNA1 encompassing the nuclear localization signal, whereas clones of EBNA1 devoid of the nuclear localization signal failed to bind to karyopherin $\alpha 2$. The interaction was biochemically confirmed by far-Western analysis using bacterially expressed karyopherin $\alpha 2$ and karyopherin $\alpha 2$ -specific monoclonal antibodies. The nuclear transport of EBNA1 was impaired by expression of N-terminally truncated karyopherin $\alpha 2$. Zone velocity sedimentation in a sucrose gradient indicated that: (i) EBNA1 and Rch1 colocalize; and (ii) the association of karyopherin $\alpha 2$ with high molecular weight protein complexes might be impeded by the presence of EBNA1.

The Epstein-Barr virus (EBV)¹ is the etiological agent of infectious mononucleosis and is implicated in the induction of several malignancies including Burkitt's lymphoma, nasopharyngeal carcinoma, Hodgkin's disease, T-cell lymphoma, and polyclonal B-cell lymphoma that arise under immunosuppression (reviewed in Ref. 1). The expression of EBV-encoded proteins is most stringently restricted in primary Burkitt's lymphoma in that only EBNA1 and the nontranslated Epstein-Barr virus-encoded small nuclear RNAs are transcribed. EBNA1 binds to the viral origin of replication (oriP)(2, 3) and is the only virus protein necessary and sufficient to replicate and maintain the EBV genome in the infected cell (4). In addition, experiments using transgenic animals show that EBNA1 by itself is able to induce tumors *in vivo* (5, 6). EBNA1 is a multifunctional phosphoprotein (7) with a variety of properties, including sequence-specific binding to DNA (8, 9), formation of homodimers (10), nuclear localization (11), and stimulation of

cellular gene expression (12).

Import of proteins into the nucleus is an active process that can be divided into at least two steps: 1) binding of proteins with a nuclear localization signal (NLS) to a cytosolic NLS-receptor and translocation to the nuclear pore complex; and 2) transport of NLS-bearing proteins into the nucleus. The NLS-receptor consists of two subunits, a 60-kDa protein that is called karyopherin $\alpha 2$ (alternatively, it is called importin α in vertebrates and Srp1 in yeast) and a 95-kDa protein, called karyopherin β (also known as importin β in vertebrates and Kap95p in yeast). Karyopherin $\alpha 2$ serves as the NLS-receptor, whereas karyopherin β functions as an adapter that mediates binding to nucleoporins. Two additional proteins, GTPase Ran/TC4 and p15 (NTF2) are required for transport into the nucleus. For recent reviews about nuclear transport, see Görlich and Mattaj (13) and Hurt (14).

Burkitt's lymphoma invariably carry a chromosomal translocation involving the *c-myc* proto-oncogene (15). EBNA1 stimulates the expression of the recombination activating genes 1 and 2 (*RAG1* and *RAG2*, respectively), which are necessary for V(D)J recombination in B-cells (16). The presence of EBNA1 in B-cells and the concomitant stimulation of *RAG1/RAG2* expression might ultimately lead to the chromosomal changes observed in Burkitt's lymphoma. Karyopherin $\alpha 2$ was originally identified and molecularly cloned by virtue of its ability to bind to the recombination activating protein 1 (*RAG1*) using the yeast two-hybrid system (17, 18); therefore, it is alternatively called Rch1 (*Rag cohort1*). In the present communication, we show that EBNA1 also binds to the nuclear transport protein karyopherin $\alpha 2$. For brevity, we refer to karyopherin $\alpha 2$ /importin α in the following report as Rch1.

MATERIALS AND METHODS

Cells and Antibodies—Human 293 GP cells derived from embryonal kidney cells by transformation with the adenovirus type 5 E1a protein (19) were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. B95–8 is an EBV-positive marmoset cell line (20), iB4 is a human lymphoblastoid cell line transformed by the B95–8 virus (21), and BJAB is an EBV-negative B-cell tumor cell line from a tumor initially diagnosed as BL which, however, does not carry a chromosomal translocation involving the *c-myc* locus (22). BL41 is an EBV-negative Burkitt's lymphoma cell line (23), and BL41-P3HR1 was established by infection of BL41 with the transformation-defective P3HR1 virus (24). The BL41 cell line was a generous gift from U. Zimmer-Strobl, GSF, Munich, Germany. The B-cells were maintained in RPMI 1640 medium (Life Technologies, Inc.) supplemented with 10% fetal calf serum (Seromed), 40 IU/ml penicillin, and 50 μ g/ml streptomycin and were subcultured routinely once per week. The insect cell line SF158 (25) was kept at 27 °C in TC100 medium supplemented as above. Recombinant baculoviruses expressing EBNA1 and EBNA2 were described earlier (26).

The rat monoclonal antibodies (mAbs) 1H4 directed against EBNA1 and R3 directed against EBNA2A have been described (27, 28). The mAb pab122 directed against p53 was a generous gift from K. Roemer, Universitätskliniken, Homburg. For the production of rat monoclonal

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¹ The abbreviations used are: EBV, Epstein-Barr virus; NLS, nuclear localization signal; mAb, monoclonal antibody; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis.

antibodies against Rch1, an *EcoRI*-resistant DNA fragment encoding amino acids 116–515 of Rch1 from clone pGADGH Rch1 122 (see below) was ligated to the *EcoRI* digested vector pATH10 (29). (We note that the *EcoRI* site at codon 515 in clone Rch1-122 was not predicted by the original cDNA sequence; the mutation leading to the new restriction site did not change the amino acid composition of Rch1 in our clone Rch1-122). The resulting plasmid pATH-Rch1 was used to express the amino acids 116–515 of Rch1 fused in-frame to the Trp E protein in *Escherichia coli* strain BL21/DE 3 (lon⁻)(30). This particular strain of *E. coli* was chosen for the expression using the pATH vectors because the absence of protease resulted in a higher yield of fusion proteins. The gel-purified protein was used to immunize Lou/c rats. The fusion and screening for Rch1-specific monoclonal antibodies was done exactly as described (28) using an irrelevant Trp E fusion protein as a control. The Rch1-specific clone 2G7 (IgG2a) was subcloned and used for additional experiments.

Plasmid Constructions—For a review of the yeast “two-hybrid” system, see Phizicky and Fields (31). The GAL4 DNA-binding domain (GAL4DBD) fusions were constructed in the vector pGBT9 (32). GAL4 DBD EBNA1 CT (330–641) was constructed by polymerase chain reaction (PCR)-mediated amplification of the DNA fragment encoding amino acids 330–641 of EBNA1, using oligonucleotide primers EBNA1 Eco (5′-CCGGAATTCGGTGGAGGAGGCAGTGGAGGC) and EBNA1 Sal (5′-CACGCCAGCTGTCACTCTGCCCTTCTCACC).

The amplified fragments were digested with *EcoRI* and *SalI* (underlined in the primers EBNA1 Eco and EBNA1 *SalI*, respectively) and ligated to *EcoRI/SalI*-digested pGBT9. Additional fragments of EBNA1 were inserted into the vector pGBT9 as described above. The amino acid encoded by the various PCR products are given in parentheses.

The primers used for the PCR amplification of EBNA1 cDNA fragments are: GAL4 DBD EBNA1 Eco (330–494), EBNA1 Eco, and EBNA1 1480R (5′-CACGCGTCTGACTCACCTAGCCAGGAGAGCTCTTAA); GAL4 DBD EBNA1 Sal (483–529), EBNA1 1450F (5′-CCGGAATCTCTAAGAGCTCTCCCTGGCT), and EBNA1 Sal; GAL4 DBD EBNA1 CT/NLS (410–529), EBNA1 NLS5′ (5′-TGTTAGGGGAATTCGATTATT), and EBNA1 Sal; and GAL4 DBD EBNA1 NLS (330–423), EBNA1 Eco, and EBNA1 NLS3′ (5′-AGGCTCACCAGCTGGGCCAC).

Plasmids pGADGH Rch1 122 and pGADGH Rch1 142 encoding amino acids 116–529 and 38–529 of Rch1, respectively, were isolated during the yeast two-hybrid screening. For the expression in *E. coli* of the full-length Rch1 protein, which consists of 529 amino acids, the complete cDNA of Rch1 was PCR-amplified from the HeLa cDNA library originally used for the two-hybrid screen using the oligonucleotide primers: N-term Rch1, 5′-GCTCTAGAGGATCCCTCATACATATGTCCACCAACGAGAATGCTAAT; and C-term Rch1, 5′-CGCGGATCCAGCTTCTTAAAGTTAAAGTCCCAGG).

The resulting DNA fragment was digested with *NdeI* and *BamHI* (underlined in the above primers) and ligated to the vector pET3a (33) to yield vector pET3a-Rch1, which directs the expression of Rch1 as a non-fusion protein in *E. coli*. The anticodon CTA, which corresponds to the TAG stop-codon of Rch1, is emphasized in boldface.

Plasmid pCEP4 R for the eukaryotic expression of an N-terminally truncated Rch1 was constructed using the PCR primers: Rch1-C-5′, 5′-CGCGGATCCCCCGGGAATTCATAACCATGGATGCTGTTGAGCAGATT; and Rch1-C-3′, 5′-CGCGGATCCCAAGCTTCTTAAAGTTAAAGTCCCAGG. The resulting DNA fragment encoding amino acids 251–529 of Rch1 was digested with *BamHI* (underlined) and ligated to vector pCEP4 (Invitrogen). The primer Rch1-C-5′ encodes an ATG initiator methionine codon (boldface) to allow the expression of the truncated Rch1 in eukaryotic cells.

Yeast Two-hybrid Screening—Yeast transformation was performed according to the method of Schiestl and Geitz (34). The yeast strain HF7c (35) was transformed with plasmid construct GAL4 DBD EBNA1 CT (330–641), and transformants were plated on Trp⁻ selective-defined media. The GAL4 DBD EBNA1 CT (330–641) transformant was subsequently transformed with a HeLa cDNA library (Clontech), and selection was done on Trp⁻Leu⁻His⁻ selective-defined media as described previously (36). Colonies that showed moderate to intense growth were streaked on Trp⁻Leu⁻His⁻ selective-defined media and tested for β -gal expression by a colony lift filter assay (37). Library-derived plasmids were recovered by transformation of competent bacteria with total yeast DNA, followed by selection for ampicillin resistance as described previously (38).

Zone Velocity Sedimentation—Extracts of 1.5×10^7 EBV-positive and EBV-negative cells were separated by centrifugation through a 15–30% sucrose gradient as described (39). Fifteen fractions collected by bottom puncture were precipitated in ethanol and subjected to 10% SDS-PAGE (polyacrylamide gel electrophoresis) followed by immuno-

blotting with mAbs 2G7 and 1H4 directed against Rch1 and EBNA1, respectively.

Transfections and Subcellular Fractionation—Transfections were performed by the calcium phosphate method, as described previously (40). After 48 h, transfected cells were fractionated. Cytoplasmic and nuclear fractions were analyzed by Western blot with anti-EBNA1 and anti-Rch1 mAb. To determine the subcellular localization of EBNA1 and Rch1, transfected 293 GP cells were lysed by Dounce homogenization exactly as described by Niedermann *et al.* (41). The nuclear and cytosolic fractions were analyzed with the EBNA1-specific mAb 1H4 and the Rch1-specific mAb 2G7.

Far-Western blotting—The association of EBNA1 with Rch1 was analyzed by a modified far-Western blotting procedure (42, 43). Extracts from baculovirus-infected SF158 cells were prepared by suspending 1×10^7 SF158 cells infected with wild-type or recombinant baculovirus expressing either EBNA1-bac or EBNA2-bac in 0.5 ml lysis buffer (26). Cells were suspended, sonicated, and boiled in SDS-gel loading buffer. After centrifugation ($13,000 \times g$, 4 °C, 10 min), extracts derived from 1×10^6 cells/gel track were separated by SDS-PAGE and transferred to nitrocellulose (Schleicher and Schuell) in a buffer that consisted of 25 mM Tris/HCl, 0.195 M glycine, pH 8.8. The membranes were preincubated for 30 min in HBB (HEPES binding buffer: 20 mM HEPES, pH 7.5, 5 mM MgCl₂, and 1 mM KCl) that contained 5% nonfat dried milk. The membrane was then incubated for 18 h at 4 °C with 0.05–0.1 ml/cm² HBB supplemented with 5% nonfat dried milk and 5 mM dithiothreitol that contained 0.1 ml of pET-Rch1 extract/ml HBB. All subsequent incubation steps were carried out in PBS, 5% nonfat dried milk, and intermittent washing steps were performed with PBS/0.1% Triton X-100. Bound Rch1 was visualized by incubating the membrane with mAb 2G7 at a dilution of 1:20, followed by incubation with peroxidase-coupled goat-anti-rat antibody (Sigma) at a dilution of 1:500.

RESULTS

Identification of Karyopherin $\alpha 2$ /Rch1 as a Cellular Target of EBNA1—EBNA1 varies considerably in length between different EBV isolates due to heterogeneity in the so-called “glycine-alanine” repeats present in the N-terminal half of the molecule (see Fig. 1). The glycine-alanine repeats were shown to down-modulate the T-cell response against EBV-infected cells (44). The remaining functions of EBNA1 are mostly attributable to sequences in the C-terminal part of the protein (11), as outlined in Fig. 1. For screening in the yeast two-hybrid system, a DNA fragment of the strain B95–8 encoding amino acids 330–641 EBNA1 obtained by PCR amplification was fused in-frame to the DNA-binding domain of GAL4 in the “bait”-vector pGBT9. The extent of this fragment and of additional, shorter C-terminal fragments of EBNA1 that were generated are shown Fig. 1. We then screened 7×10^6 individual clones of a HeLa cDNA library for the presence of interacting partner proteins expressed as fusion proteins with the GAL4-transactivation domain. Colonies that grew under selection were segregated and rescreened, and a total of 11 positive clones were obtained. Further analysis of these clones revealed that they either contained an insert of 2.0 or 2.1 kilobases; two representative isolates designated clones 142 and 122, respectively, were selected for further analysis. By DNA sequencing, we determined that the cDNA inserts encoded either amino acids 38–529 or 116–529 of the previously described *Rch1* gene, which is predicted to encode a protein of 529 amino acids (17, 18). Because the NLS was shown to encompass amino acids 379–386 of EBNA1 (11), we constructed additional bait-vectors that either did or did not contain the NLS of EBNA1, as outlined in Fig. 1. When the bait vector expressed amino acids 330–423 or 330–494 as a fusion protein with the GAL4 DNA-binding domain, the resulting proteins interacted with the corresponding Rch1 proteins from the cDNA clones obtained during the initial screen, whereas bait vectors encompassing either amino acids 410–641 or 483–641 did not yield positive signals (data not shown). These results are in line with the

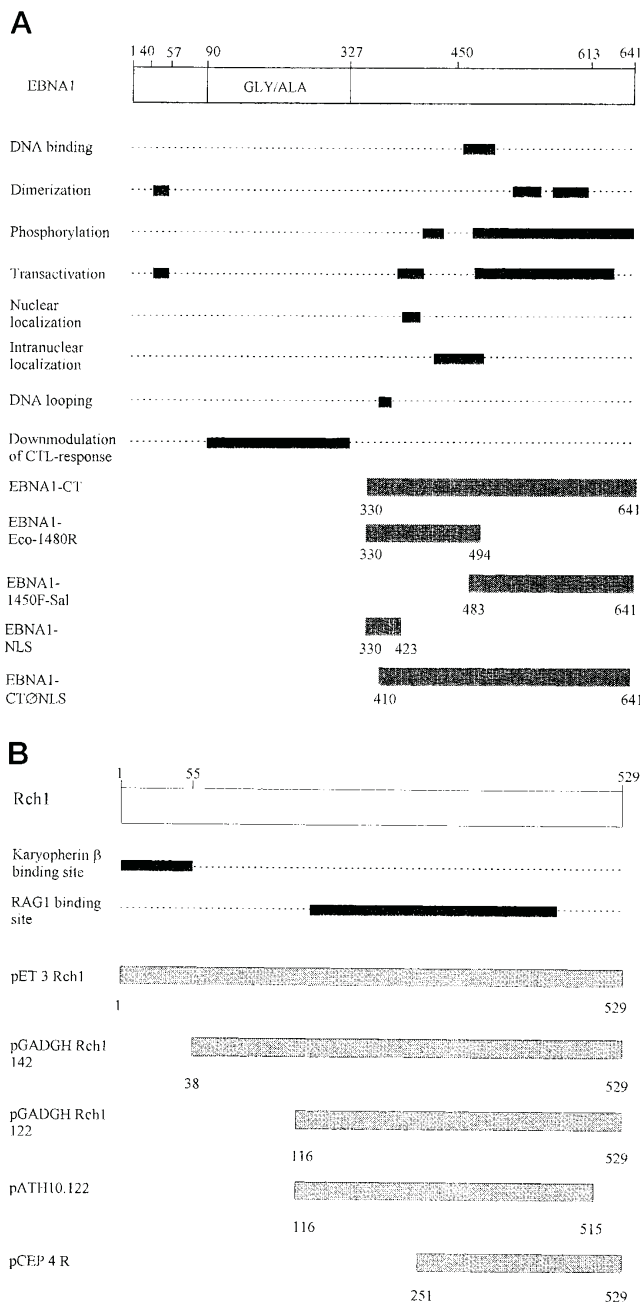


FIG. 1. A, functional domains of EBNA1. The standard EBNA1 protein, which consists of 641 amino acids (Baer *et al.* (57)) is shown at the top. The various domains as described by Ambinder *et al.* (11) are depicted in black. Down-modulation of the cytotoxic T-cell (CTL) response against EBV-infected cells by the glycine-alanine repeat (GLY/ALA) was described by Masucci *et al.* (44). The NLS of EBNA1 comprises amino acids 379–386. The fragments of EBNA1 used in the yeast “two-hybrid” system are shown in gray. The numbers indicate the amino acids residues of EBNA1 encoded by the various constructs. **B**, functional domains of karyopherin $\alpha 2$ /Rch1. The Rch1 protein, which consists of 529 amino acid residues, is shown at the top. The functional domains of Rch1 as described by Gallay *et al.* (45) and Görlich *et al.* (48) are shown in black. The various parts of Rch1 expressed in different constructs are shown in gray. The numbers indicate the Rch1-specific amino acid residues encoded by the different constructs.

observation that the NLS of EBNA1 resides between amino acids 379 and 386.

We also carried out the converse experiment to test which region of Rch1 is responsible for binding to EBNA1. A DNA fragment encoding amino acids 116–515 of Rch1, which was used for the production of monoclonal antibodies against Rch1,

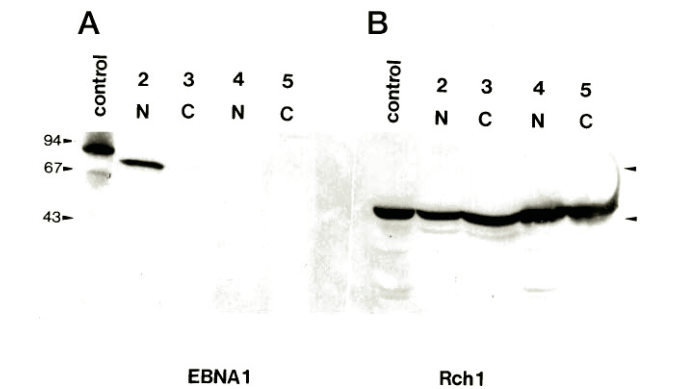


FIG. 2. Subcellular localization of EBNA1 and Rch1. **A**, the nuclear and cytosolic fractions of the EBV-positive cell line Jijoye (lanes 2 and 3) or the EBV-negative cell line BJAB (lanes 4 and 5) were separated by SDS-PAGE and transferred to the membrane and stained with the EBNA1-specific mAb 1H4. Lane 1 contains whole-cell extract of insect cells infected with a recombinant baculovirus expressing EBNA1. **B**, the extracts described in **A** were tested with the Rch1-specific mAb 2G7. The position of the molecular mass marker proteins (in kDa) is indicated by arrows on the left of **A**. The marker proteins were, in descending order: phosphorylase *b*, bovine serum albumin, ovalbumin, and carbonic anhydrase (Pharmacia Biotech Inc.). The arrows on the left of **B** indicate the position of phosphorylase *b* and bovine serum albumin.

was inserted into the vector pGAD424. We then tested this construct with the original, EBNA1-expressing bait vector in the two-hybrid system. We obtained positive signals (data not shown) and, therefore, conclude that the minimal region of interaction with EBNA1 comprises amino acids 251–515 of Rch1.

Preparation of Monoclonal Antibodies against Rch1—For further biochemical analysis, we generated monoclonal antibodies against Rch1. A DNA fragment encoding amino acids 116–515 of Rch1 was expressed in *E. coli* as a Trp E-fusion protein and used to immunize Lou/c rats. The subsequent production of monoclonal antibodies against Rch1 was carried out essentially as described earlier (27, 28). A clone, designated 2G7 (rat IgG2a), that reacted only with the fusion protein (data not shown) was subcloned and used in all of the experiments. Nuclear and cytoplasmic extracts from the Burkitt’s lymphoma cell line Jijoye, which contains the type 2 strain of EBV and extracts from the EBV-negative lymphoma cell line BJAB, were analyzed in a Western blot shown in Fig. 2. The EBNA1-specific antibody 1H4–1 reacted only with a protein of the predicted size in the nuclear extract of the cell line Jijoye, as shown in Fig. 2A, lane 2. In contrast, the Rch1-specific antibody stained a band with a molecular mass of approximately 58 kDa in the nuclear and cytoplasmic extracts of both cell lines. The presence of Rch1 in the cytoplasm and the nucleus of the cell lines was as expected because the present model suggests that Rch1 shuttles between the two cell compartments (13, 14).

Detection of Binding of Rch1 to EBNA1 by Far-Western Analysis—A cDNA encoding the full-length Rch1 protein was PCR-amplified from the HeLa cDNA library (Clontech) originally used for the two-hybrid screen and expressed in *E. coli* using the vector pET3a-Rch1. As can be seen in Fig. 3, the antibodies against Rch1 reacted with a protein of the expected size in the extract derived from *E. coli* transformed with the pET3a-Rch1 construct, whereas the bacterial control extract did not yield a signal. The *E. coli*-expressed Rch1 was subsequently used as a probe in the far-Western analysis (43, 44). We showed that the monoclonal antibodies used for the detection of the proteins do not cross-react. For this purpose, EBNA1 and Rch1 from bacteria and insect cells, respectively, were analyzed in a standard Western blot experiment shown in Fig. 3, lanes 1–4. The anti-

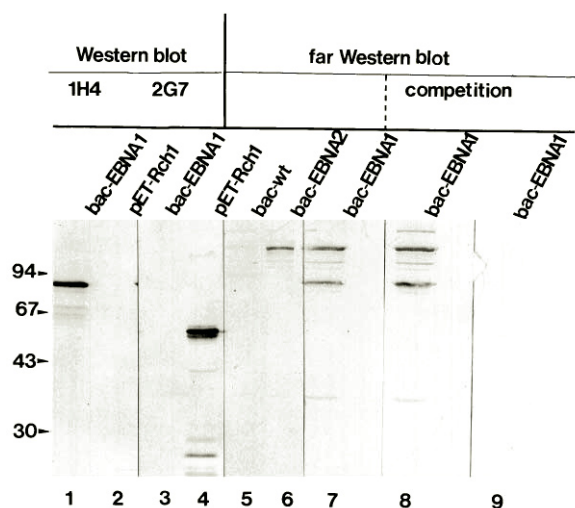


FIG. 3. Binding of Rch1 to EBNA1 as detected by far-Western analysis. EBNA1 expressed in insect cells using a recombinant baculovirus (26) (lanes designated *bac-EBNA1*) and Rch1 expressed in *E. coli* (lanes designated *pET-Rch1*) were analyzed by Western blotting using the EBNA1-specific mAb 1H4 or Rch1-specific mAb 2G7. For the far-Western analysis, extracts of insect cells infected either with wild-type baculovirus (lane designated *wt-bac*), an EBNA2A-expressing baculovirus (lane designated *bac-EBNA2*), or the EBNA1-expressing baculovirus (lanes designated *bac-EBNA1*) were separated by SDS-PAGE, transferred to nitrocellulose, and incubated with bacterially expressed Rch1 as a probe. Bound Rch1 was visualized using the mAb 2G7. In the competition experiment, the mAb 2G7 was preincubated either with bacterial extract containing either no Rch1 (lane 8) or recombinant Rch1 (lane 10). The marker proteins (in kDa) were, in descending order: phosphorylase b, bovine serum albumin, ovalbumin, and carbonic anhydrase (Pharmacia Biotech Inc.). The arrow on the right side of the figure shows the position of the EBNA1 protein.

body 1H4-1 reacted only with EBNA1 (Fig. 3, lanes 1 and 2), whereas the antibody 2G7 reacted only with Rch1 (Fig. 3, lanes 3 and 4). For the far-Western experiment, EBNA1 expressed in insect cells using a baculovirus construct (26) was separated by SDS-PAGE, transferred to nitrocellulose membrane, and incubated with *E. coli* extract containing the Rch1 probe. Bound Rch1 was visualized with mAb 2G7. Extracts from insect cells infected with wild-type baculovirus (Fig. 3, lane 5) or with a baculovirus expressing the EBV-encoded nuclear antigen 2A (26) (Fig. 3, lane 6) served as negative control. The presence of EBNA2 in the insect cell extract was confirmed separately by Western blot analysis (data not shown). Although we observed some binding of Rch1 to cellular proteins in the extract from wild-type or EBNA2-containing extract, we could clearly detect binding of Rch1 to EBNA1 (Fig. 3, lane 7). The position of EBNA1 in Fig. 3 is indicated by an arrow. In the control extracts, no binding to a protein of the same size as EBNA1 was detectable. Rch1 did not bind to EBNA2, which migrates with an apparent molecular weight of 85 kDa. As an additional control, *E. coli* extract from cells transformed with the parental pET3a plasmid (Fig. 3, lane 8) or from cells expressing Rch1 (Fig. 3, lane 9) was used to preincubate the Rch1-specific antibody 2G7. As can be seen, preincubation with control extract did not change the signal, whereas preincubation with Rch1 abolished binding to the membrane.

The nuclear localization signal of EBNA1 comprises amino acids 379–386, and we assume that the electrophoresis and transfer to the membrane does not significantly alter this sequence, which is still recognizable by native Rch1 in the far-Western analysis. However, when we carried out the reverse experiment by transferring Rch1 to the membrane and probing the blot with EBNA1 from insect cells, we were unable to detect binding of EBNA1. The region of Rch1 responsible for binding

to EBNA1 might be larger than the NLS of EBNA1 and may also be conformation-dependent. Thus, the denaturing conditions during SDS-gel electrophoresis may permanently destroy the tertiary structure of the protein, precluding binding of the soluble EBNA1 probe.

We also asked whether Rch1 would yield a signal with EBNA1 from B cells. When we carried out a far-Western experiment analogous to the one described above, we did not observe binding to the EBNA1 from B cells. We assume that the failure to observe binding was due to the low amount of EBNA1 present in B cells as compared to the baculovirus-infected insect cells. Alternatively, the EBNA1 protein from the B cells might be modified after the transport to the nucleus. This modification might inhibit the rebinding of Rch1 to EBNA1. However, because we were able to show an association of EBNA1 with Rch1 by: (i) inhibition of nuclear transport of EBNA1 by expression of an N-terminally truncated Rch1; and (ii) colocalization of the two proteins in a sucrose gradient (see below), this matter was not further pursued.

Inhibition of Nuclear Transport of EBNA1 by N-terminally Truncated Rch1—It was shown recently that the matrix protein of the human immunodeficiency virus (HIV) is transported to the nucleus by Rch1, and that translocation of the matrix protein to the nucleus is impaired by expression of the C-terminal amino acids 244–529 of Rch1 (45). From other lines of experimentation, it is clear that Rch1 binds to the protein(s) to be transported to the nucleus through its C-terminal portion, whereas the N-terminal amino acids 1–55 tether the resulting protein complex to karyopherin β , which is responsible for the transport of the resulting complex to the nuclear pore complex (46, 47). Deletion of the N-terminal part of Rch1 thus precludes binding to karyopherin β (48). In addition, the truncated Rch1 competes with the full-length Rch1 for binding to the target proteins and inhibits their nuclear translocation. Therefore, we decided to carry out a similar experiment to inhibit nuclear transport of EBNA1 with a truncated Rch1. A DNA fragment encoding amino acids 251–529 fused to an initiator methionine start codon was generated by PCR amplification and inserted into the vector pCEP 4. This vector allows the episomal replication of the plasmid by supplying the latent origin of replication of EBV together with EBNA1, which in turn induces the replication by binding to oriP of EBV; the vector directs the expression of an additional gene driven by the CMV immediate-early promoter. The resulting vector, pCEP 4 R, expressing both EBNA1 and the truncated Rch1, was transiently introduced into the cell line 293 GP, which allows high level expression of the vector-encoded proteins by calcium phosphate precipitation. Forty-eight h after the transfection, the 293 GP cells were fractionated and analyzed by Western blotting. As shown in Fig. 4, we could detect the truncated Rch1 protein in addition to the full-length, cellular Rch1 in the cytoplasmic fraction (Fig. 4, lane 10) but not in the nuclear fraction of 293 GP cells transfected with vector pCEP 4 R (Fig. 4, lane 9). In contrast, the full-length Rch1 was detectable in both the cytoplasmic and the nuclear fraction as predicted. When we used the parental vector pCEP 4, we only observed the full-length Rch1 in both cell compartments (Fig. 4, lanes 7 and 8). The same extracts were then tested for the distribution of EBNA1. Using the parental vector, we could detect approximately equal amounts of EBNA1 in both the cytoplasm (Fig. 4, lane 2) and the nucleus (Fig. 4, lane 3), probably because a very high level of protein expression was induced. In contrast, we found that the cytoplasmic extracts derived from cells expressing the truncated Rch1 contained at least 80% of EBNA1, with the remaining portion detectable in the nucleus (Fig. 4, lanes 5 and 4, respectively). To ensure that the unexpectedly large amount of

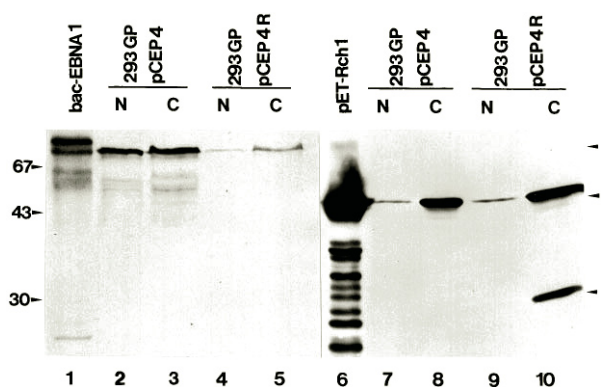


FIG. 4. **N-terminally truncated Rch1 inhibits nuclear import of EBNA1.** Human 293 GP cells were transfected with control plasmid pCEP 4 expressing EBNA1 and plasmid pCEP 4 R expressing both EBNA1 and truncated Rch1. The truncated Rch1 consists of amino acids 251–529 of Rch1. The cytosolic and nuclear fractions (lanes designated C or N, respectively) of cells transfected with the parental vector (lanes designated 293 GP, pCEP 4) or the vector expressing the truncated Rch1 (lanes 293 GP, pCEP 4 R) were analyzed by Western blot analysis using the EBNA1-specific mAb 1H4 (lanes 1–5) or the Rch1-specific mAb 2G7 (lanes 6–10). Extract from insect cells expressing recombinant EBNA1 (lane 1) or bacterially expressed Rch1 (lane 6) served as a control. The marker proteins were as in Fig. 1; the positions of EBNA1, full-length Rch1, and truncated Rch1 are indicated by arrows on the right side of the figure.

EBNA1 detected in the cytoplasmic fraction was not due to an experimental error, we also analyzed the same fractions for the presence of p53, which is a nuclear protein. The major portion of p53 was detectable in the nuclear fraction with about 10–15% of total p53 detectable in the cytoplasmic fraction, which might constitute the freshly synthesized p53. We conclude from this observation that the nuclear transport of EBNA1, but not of p53, is specifically mediated by Rch1. In addition to the retention of EBNA1 in the cytoplasmic fraction, we also observed a reduction in the overall expression of EBNA1 (Fig. 4, compare lanes 2 and 3 with lanes 4 and 5). We take this as an additional indication that the specific nuclear transport of EBNA1 by the wild-type Rch1 is inhibited by the truncated Rch1. The reduced amount of EBNA1 transported to the cell nucleus ultimately results in a strongly reduced rate of the (EBNA1-dependent) replication rate of the vector pCEP 4 R, which in turn yields lower levels of EBNA1 protein.

The Complex Formation of Rch1 Is Altered in the Presence of EBNA1—We wanted to test whether the presence of EBNA1 in B-cells interfered with the formation of complexes between Rch1 and cell proteins. For this purpose, cell extracts were analyzed by sucrose gradient centrifugation as described previously (39). We compared the EBV-negative cell lines BL41 and BJAB (both cell lines were isolated from patients diagnosed with Burkitt's lymphoma) with the EBNA1-expressing cell lines BL41-P3HR1 (BL41 cells infected with the P3HR1 strain of EBV) and the human lymphoblastoid cell line iB4 (generated from uninfected primary human lymphoid cells by infection with the B95–8 virus). The results obtained with the cell lines BJAB and iB4 are shown in Fig. 5. The position of molecular mass standard proteins separated on a gradient, which was analyzed in parallel, is indicated above the top panel; the lanes designated “U” contain unfractionated whole-cell extract as a control. As can be seen in the top panel, the major part of Rch1 from EBV-negative B-cell line BJAB was found in the top of the gradient (fractions 10–15), corresponding to uncomplexed, monomeric Rch1 (fractions 13–15) or low molecular mass complexes (fractions 10–12). A smaller but sizable fraction of Rch1, however, was detectable in fractions 1–5 corresponding to very high molecular mass complexes. In

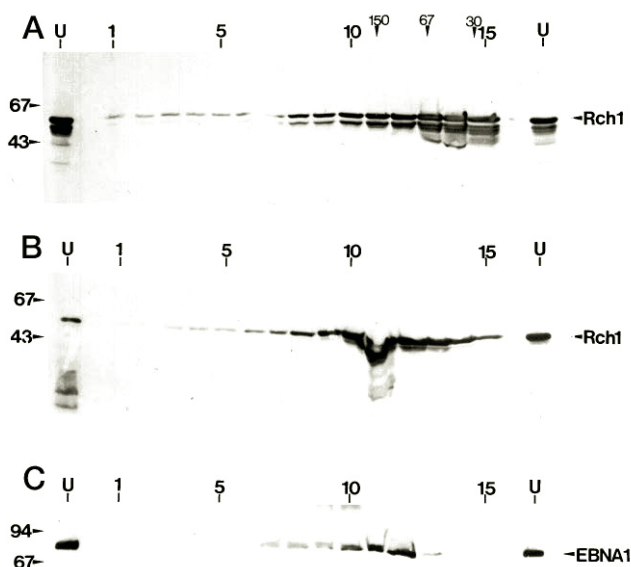


FIG. 5. **Zone velocity sedimentation of Rch1 protein and EBNA1 protein.** Extracts of EBV-negative cells, BJAB (A), and EBV-positive cells, iB4 (B and C), were separated by centrifugation through a 5–30% sucrose gradient (39). Fifteen fractions (1, bottom: 30%; 15, top: 5%) were collected and were subjected to 10% SDS-PAGE followed by immunoblotting with anti-Rch1 mAb (A and B) and anti-EBNA1 mAb (C). Sedimentation standards (29 kDa, fraction 15; 67 kDa, fraction 13; 150 kDa, fraction 11) are indicated by arrowheads above panel (A). U, unfractionated cell extract. The positions of molecular mass marker proteins (in kDa, see Fig. 1) and of Rch1 and EBNA1 are indicated.

contrast, when cell extract derived from the EBV-positive iB4-cell line was analyzed, we found that Rch1 had a different migration behavior (Fig. 5, middle panel). The major portion of Rch1 was now detectable in fractions 10–12 instead of fractions 13–15, corresponding to a molecular mass of approximately 150–250 kDa. When the fractions from the same gradient were tested for the presence of EBNA1 (Fig. 5, bottom panel), we found that the peak of EBNA1 reactivity was also detectable in fractions 10–12, indicating that Rch1 is in a complex with EBNA1. EBNA1 and Rch1 have calculated molecular weights of M_r 72,000 and M_r 58,000, respectively. Assuming that the majority of the EBNA1 forms homodimers (49), this result would indicate that dimeric EBNA1 binds either one or two molecules of Rch1, resulting in a molecular mass of about 200 kDa. In our experiments, we observed a molecular weight of approximately M_r 150,000–200,000. It is also possible, however, that monomeric EBNA1 binds to Rch1.

We obtained the same results with the cell lines BL41 and BL41-P3HR1 (data not shown). In the BL41-P3HR1 cells, we found a lower amount of EBNA1 as compared to iB4. In BL41-P3HR1, we also noted a shift of the low molecular weight form of Rch1 to the higher molecular weight fractions that coincided with the EBNA1-containing fractions. We also noted a slight reduction of the very high molecular weight form of Rch1.

The result that Rch1 and EBNA1 colocalize in conjunction with the results obtained from the far-Western analysis and the fact that the truncated Rch1 impedes the import of EBNA1 into the nucleus further strengthens the notion that the proteins form a complex. Furthermore, we reproducibly observed a reduction in the amount of Rch1 detectable in fractions 1–5 corresponding to high molecular mass complexes. There are two possible explanations for this result. Either the presence of EBNA1 prevents the binding of Rch1 to high molecular mass

complexes, or EBNA1 inhibits the synthesis of proteins contained in those complexes.

DISCUSSION

Transformation of B lymphocytes *in vitro* by the Epstein-Barr virus is mediated through a set of viral proteins that are found in the nucleus or the membrane of the cell. By genetic means, it was shown that the nuclear antigens EBNA1, -2, -3A, -3C, and -5 as well as the latent membrane protein 1 are absolutely essential for this transformation process (reviewed in Ref. 50). It is generally assumed that viral oncogenes bind to cellular factors that regulate cell growth, thereby changing their biochemical and biological activities; for instance, binding of the multifunctional SV40 large T-antigen to the p53 and p105rb proteins is thought to induce the molecular changes resulting in cell transformation (reviewed in Refs. 51). For EBNA-2, a variety of cellular partners, mostly proteins involved in gene regulation, have been identified; EBNA-5 appears to target the p53 and p105^{rb} proteins, and latent membrane protein 1 forms a complex with the tumor necrosis factor receptor family of growth factor receptors (52). Thus far, no cellular proteins that might be targeted by EBNA1 to carry out its various functions have been described. We have used the yeast two-hybrid system to identify such cellular proteins to gain a deeper understanding of the EBV-mediated transformation. Here we show that EBNA1 binds to karyopherin $\alpha 2$, which is also called importin α or Rch1. To our knowledge, this is the first description of such a cellular partner protein of EBNA1.

Karyopherin $\alpha 2$ is involved in the nuclear import of proteins (13, 14). By another line of experimentation, it was shown that a protein termed Rch1, which is identical to karyopherin $\alpha 2$, binds to the recombination activation protein RAG1 (17, 18). Furthermore, it was shown that the presence of EBNA1 in B lymphocytes induces the expression of the recombination activating genes *RAG1* and *RAG2* (16). The proteins, in turn, are necessary and sufficient to induce the V(D)J-recombination, which is a crucial step in B-cell maturation. However, the same mechanism leading to the rearrangement of the immunoglobulin loci in B cells might be active during the chromosomal translocation involving the immunoglobulin loci and the *c-myc* gene, which ultimately leads to the genesis of Burkitt's lymphoma (53). At this point, it remains unclear whether the transport of both RAG1 and EBNA1 by the nuclear transporter Rch1 and the stimulation of RAG1 (and RAG2) expression by EBNA1 is coincidental. EBNA1 has an intrinsic property to bind to DNA, and it is known that EBNA1 can juxtapose segments of DNA that are otherwise apart from each other (49, 54). It is conceivable that EBNA1 might be involved in the formation of DNA complexes that undergo illegitimate recombination as observed in Burkitt's lymphoma. This recombination event in turn might be induced by the EBNA1-stimulated expression of RAG1 and RAG2.

One point of concern was whether the observed binding of EBNA1 to Rch1 was specific or just simply the result of a fortuitous binding due to the presence of a possibly "promiscuous" nuclear localization site in EBNA1. We assume the latter not to be the case because the EBNA1 bait used for the screening in the yeast two-hybrid system did not react with the control vector pTD1. This construct encodes the GAL4-activation domain fused to amino acids 84–708 of the SV40 large T antigen, which encompasses the NLS of large T antigen. Also, when using the complete *EBNA-3C* gene instead of the EBNA1 as a bait, we observed no binding to Rch1 in the

yeast two-hybrid assay.² By definition, EBNA3C is a nuclear antigen encoded by EBV that must contain an NLS. In addition, we also did not observe binding of Rch1 to the nuclear antigen EBNA-2 in a far-Western experiment, whereas we observed strong binding to EBNA1. Lastly, the expression of a truncated Rch1 in 293 GP cells specifically impeded the nuclear import of EBNA1 but not of p53. We, therefore, deem it unlikely that the observed binding of the nuclear protein EBNA1 to the nuclear transporter Rch1 is purely artifactual.

Finally, we found that Rch1 migrated with different mobilities during sucrose gradient centrifugation, depending on the presence or absence of EBNA1. In the EBNA1-negative cell lines BJAB and BL41, Rch1 was localized mainly in low molecular weight fractions corresponding to uncomplexed, monomeric Rch1 but also in fractions corresponding to very high molecular weight. In the EBNA1-positive cell lines iB4 and BL41-P3HR1, the low molecular weight Rch1 was shifted to the EBNA1-containing fractions with intermediate molecular weight, whereas the amount of Rch1 in the complexes with very high molecular weight was reduced. This latter effect was more pronounced in the iB4 cells that expressed more EBNA1 than the BL41-P3HR1 cells. These results indicate that EBNA1 might inhibit binding to the high molecular weight complexes by a squelching mechanism. Although the reduction of high molecular weight complexes was less pronounced, we clearly observed a shift of the Rch1 protein in the low molecular weight fractions to a fraction of intermediate molecular weight, which also contained EBNA1. The presumed complex formation would then prevent binding to other proteins present in the cells. From the Western blot experiment, we estimated that the amount of Rch1 in the cell is at least 50-fold higher than the amount of EBNA1, but the signals obtained with the different antibodies might not reflect the true amount of each protein in the cell. However, the shift in Rch1 mobility might be due to the fact that exactly the portion of the Rch1 molecules that are otherwise uncomplexed are now bound by EBNA1. Ultimately, the presence of EBNA1 appears to reduce the binding of Rch1 to cellular factors, either by directly binding to Rch1 or by a qualitative change in the expression of these proteins. It is tempting to speculate that the reduced binding of Rch1 to such proteins might impede their import to the nucleus, thus preventing their activity. These proteins could be involved in controlling the proper execution of the recombinatorial events during B-cell maturation. In this respect, it is noteworthy that the *oho-31* tumor suppressor gene in *Drosophila melanogaster* is a homologue of Rch1. The inactivation of *oho-31* leads to tumors of the hematopoietic organs and the genital disk in the fly (55). The *oho-31* gene product is predominantly cytosolic in interphase but strongly accumulates in the nucleus during mitosis. Of the latent gene products of EBV, EBNA1 is the only protein that is found to be associated with the chromatin during mitosis (56) and is thought to ensure the equal distribution of the viral DNA to the progeny cells. At this point, we assume that the binding of EBNA1 to Rch1 results in a quantitative change of the Rch1 protein available for nuclear transport that is not available for transport of its normal partner proteins. Additional experiments will be carried out to clarify whether the specific transport of EBNA1 by Rch1 is necessary for EBV-mediated transformation or whether the nuclear transport of EBNA1 is the sole prerequisite for its function in cell transformation.

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² N. Fischer, unpublished observations.

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