Human RPA (hSSB) interacts with EBNA1, the latent origin binding protein of Epstein–Barr virus

Dan Zhang, Lori Frappier¹, Emma Gibbs², Jerard Hurwitz² and Mike O'Donnell*

Laboratory of DNA replication, The Rockefeller University, Howard Hughes Medical Institute, 1230 York Avenue, New York, NY 10021, USA, ¹Department of Medical Genetics and Microbiology, University of Toronto, 1 Kings College Circle, Toronto, Ontario M5S 1A8, Canada and ²Program in Molecular Biology, The Memorial Sloan Kettering Cancer Center, 1275 York Avenue, Box 97, New York, NY 10021, USA

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ABSTRACT

RPA is the replicative single-strand DNA (ssDNA) binding protein of eukaryotic chromosomes. This report shows that human RPA interacts with EBNA1, the latent origin binding protein of Epstein-Barr virus (EBV). RPA binds to EBNA1 both in solution, and when EBNA1 is bound to the EBV origin. RPA is a heterotrimer, and the main contact with EBNA1 is formed through the 70 kDa subunit of RPA, the subunit which binds to ssDNA. We propose that this interaction between RPA and EBNA1 is an early step in activation of the latent origin of EBV.

INTRODUCTION

Epstein–Barr virus (EBV) is the human herpes virus associated with infectious mononucleosis as well as several malignancies (1). During latent infection of human B lymphocytes, multiple copies of the EBV genome are maintained as extrachromosomal DNA plasmids which replicate once per cell cycle (2,3). Only one virally encoded protein, Epstein–Barr virus Nuclear Antigen 1 (EBNA1), is needed for latent replication of EBV genomes (4). The EBNA1 dimer binds to an 18 bp palindromic sequence which is present in multiple copies in the EBV origin of latent replication, *oriP* (5–7). *OriP* is 1.8 kb in length and contains two elements important for replication: (i) an array of 20 copies of a 30 bp sequence, each of which contains an EBNA1 binding site, and (ii) a region of dyad symmetry that contains four EBNA1 binding sites (7,8). These elements are separated by 1 kb of intervening DNA that forms a loop when EBNA1 binds to the two *oriP* elements (9,10). The origin of replication is believed to be within the dyad symmetry element (11–13), and EBNA1 induces a structural distortion in this region (14–16). Such distortion of origin DNA appears to be a general feature of origin binding proteins. Unlike the virally encoded SV40 origin binding protein, large T-antigen and the E1 protein of bovine papilloma virus (BPV), EBNA1 is not a helicase (17). Hence, EBNA1 must recruit virtually all of the host proteins needed to advance a replication fork including PCNA, RFC, the DNA polymerases, polymerase-primase, RPA and the cellular helicase.

As a start towards understanding how EBNA1 functions with other cellular factors, we have identified its interaction with RPA, the human single-strand DNA (ssDNA) binding protein (also called hSSB). Human RPA is a heterotrimer originally purified as an essential component of the SV40 *in vitro* replication system (18–20). The largest component of RPA, the 70 kDa subunit, directly binds to ssDNA (21,22). The 32 kDa subunit is phosphorylated in a cell cycle dependent manner in both yeast and humans (24) and recent evidence indicates that this subunit also directly interacts with ssDNA (41, Bochkareva, E., Frappier, L., Edwards, A.M. and Bochkarev, A., submitted). The function of the 14 kDa subunit is unknown, but the genes encoding all three subunits are essential in yeast (23). Besides binding ssDNA, RPA interacts with a variety of proteins involved in DNA metabolism, including the SV40 large T-antigen (25), the E2 protein of BPV (26), DNA polymerase α -primase (25), Epstein–Barr nuclear antigen 2 (28), VP16 (27), GAL4 (27), p53 (26) and the xeroderma pigmentosum group A protein (29). Hence, the interaction of EBNA1 with RPA could lead to recruitment of other host replication proteins needed to replicate the EBV genome.

MATERIALS AND METHODS

Materials

Labeled ribo- and deoxyribonucleotides were from Dupont-New England Nuclear; unlabeled ribo- and deoxyribonucleotides were from Pharmacia-LKB; pET3 and pET16 were from Novagen; CM carboxymethyl dextran matrix-coated sensor chips CM5 were from Biacore Inc.; Heparin Agarose was from BioRad Laboratories; ssDNA agarose was from Gibco-BRL; HiTrap chelate chromatography resin and CM Sepharose were from Pharmacia-LKB. The β -subunit sliding clamp of the replicative DNA polymerase of *Escherichia coli*, DNA polymerase III holoenzyme, was purified as described (30). The *E. coli* expressed PCNA sliding clamp of the human DNA polymerase δ holoenzyme was purified as described (31). Human RPA was purified from HeLa cells as described (32). bEBNA1 expressed in baculovirus was purified as described (17). The catalytic subunit of cAMP dependent protein kinase, produced in *E. coli*, was a gift of Dr Susan Tayler (University California at San Diego). Surface

* To whom correspondence should be addressed. Tel: +1 212 327 7251; Fax: +1 212 327 7254; Email: odonnell@rockvax.rockefeller.edu

plasmon resonance buffer is 10 mM HEPES-HCl, pH 7.4, 150 mM NaCl, and 0.005% Tween-20. Kinase buffer is 20 mM Tris-HCl, pH 7.5, 2 mM dithiothreitol, 350 mM NaCl, 12 mM MgCl₂, 10 mM NaF, 60 μ M ATP, 5 μ Ci [γ -³²P]ATP. Column buffer is 20 mM Tris-HCl, pH 7.5, 8 mM MgCl₂, 350 mM NaCl, 100 μ g/ml BSA and 5% glycerol.

EBNA1^{PK}

The polymerase chain reaction was used to produce a gene encoding the C-terminal 200 amino acids of EBNA1, followed by a six residue protein kinase motif (RRASVP; 19) (referred to in this report as EBNA1^{PK}). The EBNA1^{PK} gene was cloned into PET3c, and is expressed to 30% of the total protein in *E. coli* BL21DE3pLysS. *Escherichia coli* BL21DE3pLysS (30 l) was grown at 37°C in LB media containing 100 μ g/ml ampicillin to an OD₆₀₀ of 0.6, followed by addition of 0.4 mM IPTG and a further incubation for 2 h. Homogeneous EBNA1^{PK} (250 mg) was obtained from the cell lysate following ion exchange chromatography on Heparin-agarose, CM Sepharose and MonoQ. Protein content was determined by absorbance at 280 nm from the molar extinction coefficient calculated from the tyrosine and tryptophan content (41 820 l/mol, as dimer). EBNA1^{PK} retains its dimeric structure and is active in site specific DNA binding (Fig. 3).

Recombinant RPA

To obtain large amounts of RPA, and subassemblies of RPA, *E. coli* expression vectors were constructed. The three genes encoding the RPA heterotrimer were obtained by reverse transcriptase/polymerase chain reaction using mRNA isolated from HeLa cells. Oligo dT₂₀ was used as a downstream primer for the reverse transcriptase. For the DNA polymerase amplification, oligonucleotides were designed from the known sequence of the RPA genes (33–35). In each case, the amplified subunit genes were sequenced to ensure that no mistakes occurred during the amplification. The individual genes were each placed into pET3 precisely at the *Nde*I site encoding the initiating Met residue, and into pET16 (which places a 20 residue leader containing 10 histidines on the N-terminal Met residue). Expression of the 70 and 14 kDa genes were improved at least 5-fold by the presence of the His tags. All three subunit genes were placed into a single T7 promoter based expression plasmid containing the 32, 14^{his} and 70^{his} kDa genes, similar to a previously published report on expression of RPA in *E. coli* (36). In addition, a plasmid expressing only the 32 and 14^{his} kDa genes was constructed. Expressed complexes were purified by HiTrap chelate chromatography followed by Heparin-agarose, and MonoQ chromatography. In the case of the RPA trimer, some excess 32•14^{his} complex was observed in the final preparation. This heterodimer was removed from the trimer by an additional ssDNA agarose column. From 20 l of induced *E. coli*, ~80 mg of 32•14^{his} complex and 60 mg of the trimeric RPA (70^{his}•32•14^{his}) were obtained. Protein content was determined by the Bradford based method using Protein Stain from Bio-Rad Inc. The recombinant RPA had comparable activity to RPA isolated from HeLa cells (assayed as DNA synthesis dependent on PCNA, RFC and DNA polymerase δ ; 37).

The 70 kDa subunit of RPA is insoluble when expressed in *E. coli*. A small amount of the 70^{his} kDa subunit was obtained by

solubilizing the cell debris in 6 M urea followed by purification by HiTrap chelate chromatography using 6 M freshly deionized urea throughout the procedure. The purified 70^{his} kDa subunit was renatured in steps by dialysis against buffer (20 mM Tris-HCl, pH 7.5, 1 M NaCl, 0.5 mM EDTA, 2 mM DTT) containing decreasing amounts of urea (6, 4, 2 and then 1 M urea) followed by dialysis against this same buffer, but lacking urea and containing 350 mM NaCl. Approximately 20% of the 70^{his} kDa subunit was recovered as soluble protein.

Surface plasmon resonance

Recombinant RPA (145 ng/ml in 10 mM sodium acetate, pH 4.5) was immobilized on a carboxymethyl dextran matrix-coated sensor chip CM5 by carbodiimide covalent linkage [1520 resonance units (RU) final] according to the manufacturers' instructions (Biacore Inc.). By similar procedures the 32•14^{his} kDa complex of RPA (1510 RU final), and the 70^{his} kDa subunit (1080 RU final) were immobilized. Solutions of EBNA1^{PK} were dialyzed against surface plasmon resonance buffer to reduce buffer related artifacts. Solutions (30 μ l) containing the indicated concentrations of bEBNA1 were then passed over immobilized RPA at a flow rate of 5 μ l/min in surface plasmon resonance buffer. EBNA1^{PK} has insignificant (<1% of signal) non-specific association with the sensor chip under these conditions. After 6 min of injection, surface plasmon resonance buffer lacking EBNA1^{PK} was passed over the chip. After completing each analysis in which EBNA1^{PK} was passed over immobilized RPA, the surface of the chip was regenerated by injection of 10 μ l of 0.1 M glycine, pH 9.5 which released non-covalently bound protein without decreasing the capacity of the immobilized protein to bind EBNA1^{PK} in future injections. Negative controls were: (i) human PCNA (30 μ l of 1 μ M solution in surface plasmon resonance buffer) was injected over the immobilized RPA, and (ii) human PCNA was immobilized on the sensor chip (1800 RU final) and a 1 μ M solution of EBNA1 was passed over the immobilized PCNA.

EBNA1^{PK} interaction with the dyad symmetry element

Gel mobility shift assays were performed using 100 fmol gel purified dyad symmetry element that was end-labeled with [α -³²P] (described below), and different amounts of EBNA1^{PK} (0, 28, 140, 280, 560, 840 and 1120 fmol) in 25 μ l of 20 mM Tris-HCl, pH 7.5, 8 mM MgCl₂, 0.1 mg/ml BSA, 350 mM NaCl and 5% glycerol. Reactions were incubated for 15 min at 23°C, then 25 μ l of each reaction was loaded onto a 4% native polyacrylamide gel. Gels were dried and exposed to X-ray film. The 263 bp section of DNA containing the dyad symmetry element used in gel mobility shift experiments was prepared as follows. A 140 bp section of DNA containing the dyad symmetry element was excised from pGEMoriP (17) using *Eco*RV (cuts at EBV map position 9047) and *Hpa*I (cuts at EBV map position 9186). The resulting fragment was gel purified, and ligated into the pGEM-BEND plasmid (Sankar Adhya) which was first cut with *Xba*I and *Sal*I followed by treatment with *Poll* and dCTP, dGTP, dATP and TTP to fill in the ends. The resulting plasmid was digested with *Xho*I and the resulting 263 bp fragment (the dyad symmetry element is flanked on both sides by nucleotides from the pGEM BEND plasmid) was gel purified and then radiolabeled using Klenow fragment and [α -³²P]dCTP to fill in the ends (specific activity 4827 c.p.m./fmol).

Analysis of the stoichiometry of EBNA1^{PK} bound to the dyad symmetry element was performed upon mixing 190 pmol [³²P]EBNA1^{PK} (21 c.p.m./fmol), with 35 µg (17.1 pmol) pGEMdyad (9) in 162 µl of column buffer. The reaction was incubated at 23°C for 15 min and then applied to a column containing 5 ml of BioGelA15m (Bio-Rad Inc.) resin equilibrated in column buffer. Fractions of 230 µl were collected. To measure the DNA content in each fraction, 175 µl was removed from each fraction analyzed and then diluted to 700 µl (using 525 µl of column buffer) for measurement of the absorbance at 260 nm in a Perkin Elmer (model) spectrophotometer. DNA content was quantitated using a conversion factor of 1 absorbance unit equal to 50 µg/ml of DNA. Then the sample was removed from the cuvette and the [³²P]EBNA1^{PK} was quantitated from its known specific activity by scintillation counting. An identical experiment, but containing 31.2 µg pGEM II in place of the pGEMdyad, was performed as a negative control for non-specific binding of [³²P]EBNA1^{PK} to DNA.

Kinase protection assays

EBNA1^{PK} (50 pmol as dimer) and β^{PK} (50 pmol as dimer) (39) were incubated with 1 nmol RPA with or without 25 pmol of the dyad symmetry element (25 pmol × 4 sites per dyad = 100 pmol EBNA1 binding sites) in 3 µl of 10× kinase buffer. The binding measurements of EBNA1^{PK} to the dyad symmetry element (Fig. 3) indicate that using these excess amounts of DNA all the EBNA1^{PK} should be bound to the dyad symmetry element. In kinase reactions lacking RPA, an equivalent weight (120 µg) of BSA was added in place of RPA. After 30 min at 15°C, 1 µl containing 0.0015 U of protein kinase was added and timed aliquots were removed and quenched with 40 mM EDTA and 1% SDS. Aliquots were analyzed by SDS-polyacrylamide gel electrophoresis followed by autoradiography, and quantitated using a phosphorimager. The percent phosphorylation of the EBNA1^{PK} was calculated from the known concentration of EBNA1^{PK} and the specific activity of the [^γ-³²P]ATP. In the absence of RPA, the percent of EBNA1^{PK} phosphorylated in 8 min was ~90% in the absence of DNA, and ~67% in the presence of DNA. The 140 bp dyad symmetry element, containing four EBNA1 sites, was amplified by PCR and purified from an agarose gel. The map positions of the start and end points of this dyad symmetry sequence in the EBV genome are 9047–9186.

RESULTS AND DISCUSSION

RPA interacts with EBNA1

To study the interaction of RPA with EBNA1, we constructed *E. coli* expression plasmids for efficient production of the three-subunit RPA, and for production of the two-subunit form containing only the 32 and 14 kDa subunits of RPA. Since we failed to express full-length EBNA1 in *E. coli*, we expressed and purified an N-terminal truncated version of EBNA1 (referred to in this report as EBNA1^{PK}) which contains a six residue extension at the C-terminus. This C-terminal extension serves as an efficient substrate for cAMP-dependent protein kinase thereby providing the ability to monitor interactions with RPA using the kinase protection assay.

To determine whether RPA and EBNA1 interact, we used two different techniques, surface plasmon resonance (Fig. 1) and the kinase protection assay (Fig. 2). Figure 1 shows the results of the

surface plasmon resonance study. In surface plasmon resonance, one partner of an interacting pair is coupled to a sensor chip, and then the other partner is passed over the chip (38). If the two macromolecules interact, an increase in mass is detected and is recorded in resonance units, 10 RU is approximately equal to 0.1 mg/ml of accumulated mass on the sensor chip. In Figure 1A, RPA was immobilized on the sensor chip, and then solutions of EBNA1^{PK} of various concentrations were passed over the top. The first arrow in Figure 1A (labeled EBNA1^{PK}) denotes the point at which a solution containing EBNA1^{PK} is first injected over the immobilized RPA. This injection continues for 6 min. During this injection the rising RU indicates an accumulation of mass, presumably due an increasing number of EBNA1^{PK} molecules interacting with the immobilized RPA. The final RU value depends on the concentration of the EBNA1^{PK} protein. As the concentration of EBNA1^{PK} injected over RPA is raised, the observed RU increases as well, consistent with an interaction between EBNA1^{PK} and the immobilized RPA. After the injection of EBNA1^{PK}, a second injection containing buffer that lacks EBNA1^{PK} is passed over the chip (denoted by the second arrow in Fig. 1A). During this injection the RU decreases due to the dissociation of EBNA1^{PK} from the immobilized RPA (i.e. there is a loss of the accumulated mass on the sensor chip resulting in an observed decrease in RU).

Accumulation of EBNA1^{PK} on the sensor chip depended upon the presence of immobilized RPA; accumulation was not observed using a non-derivatized chip (not shown), or using immobilized PCNA, the sliding clamp processivity factor of the human DNA polymerase δ holoenzyme (shown in Fig. 1C). The interaction was specific to EBNA1 and RPA, as other proteins (human PCNA, and the β-subunit sliding clamp processivity factor of the *E. coli* DNA polymerase III holoenzyme) did not interact with immobilized RPA (shown in Fig. 1B for PCNA). Finally, the interaction was not due to the protein kinase tag on the EBNA1^{PK}, as EBNA1 lacking the kinase motif also interacted with immobilized RPA (shown later in Fig. 3). Results similar to those described in Figure 1 were obtained using RPA isolated from HeLa cells (not shown).

We would also have liked to have performed these experiments by injecting RPA over immobilized EBNA1^{PK}. However, RPA associated non-specifically with the sensor chip at physiological pH values which precluded its use in the solution phase.

In Figure 2, the protein kinase recognition motif on the C-terminus of EBNA1^{PK} was utilized to study the interaction of EBNA1 with RPA in solution. In the kinase protection assay, the protein tagged with the kinase recognition motif is mixed with the suspected interacting protein (39). Then protein kinase and [^γ-³²P]ATP are added and timed aliquots are removed for SDS polyacrylamide gel electrophoresis. If the two proteins interact, and if the site of interaction is close to the position of the kinase recognition motif, then the rate of phosphorylation should be inhibited relative to the rate of phosphorylation of the tagged protein in the absence of the interacting partner.

In Figure 2A the kinase protection assay was performed in the absence of DNA. EBNA1^{PK} was incubated with [^γ-³²P] ATP and protein kinase in the presence or absence of RPA. As an internal control, we added a protein (β^{PK}) which contains the protein kinase motif but should not interact with RPA (the β-subunit acts as a DNA sliding clamp processivity factor for the *E. coli* DNA polymerase III holoenzyme; (39). At different times, a portion of the reaction was quenched with 20 mM EDTA and 1% SDS,

Figure 1. EBNA1 interacts with human RPA. Surface plasmon resonance analysis of EBNA1-RPA interaction. (A) Solutions of EBNA1^{PK} were passed over immobilized RPA as described in Materials and Methods. Negative controls are shown in (B) and (C). (B) Human PCNA was passed over immobilized RPA. (C) EBNA1^{PK} was passed over immobilized human PCNA. The protein in the fluid phase is indicated above the first arrow and its concentration is shown at the right of each trace. The start and end points of protein injections are shown by arrows.

followed by analysis in an SDS polyacrylamide gel. Quantitation of the autoradiogram showed that the presence of RPA decreased the rate of phosphorylation of EBNA1^{PK} by ~50% in the linear portion of the assay, supporting the conclusion that RPA interacts with EBNA1. This result further suggested that RPA interacts near the C-terminus of at least one of the protomers of the EBNA1 dimer. The result was not due to general inhibition of the protein kinase by RPA, as phosphorylation of the heterologous protein, β ^{PK}, was not inhibited (Fig. 2).

Does RPA bind EBNA1 in complex with the dyad symmetry element of the EBV origin?

To determine whether RPA associates with EBNA1 when EBNA1 is bound to the latent EBV origin, we performed the kinase protection assay using EBNA1^{PK} in the presence of the dyad symmetry element of *oriP*. The EBNA1^{PK} derivative contains the C-terminal 200 amino acids of EBNA1 and should bind DNA as a previous study has shown that stable interaction with DNA is observed with a derivative of EBNA1 consisting of

Figure 2. RPA interacts with EBNA1 bound to the EBV origin. Interaction of RPA with EBNA1^{PK} was analyzed by the kinase protection assay either in the absence of DNA (A) or presence of DNA (B). Kinase was added to EBNA1^{PK} in the presence or absence of RPA and timed aliquots were removed and analyzed by SDS polyacrylamide gel electrophoresis. *Escherichia coli* β ^{PK} was included in the reactions as an internal control. The autoradiograms are shown at the top and the quantitation using a phosphorimager is plotted below.

only 183 of the C-terminal residues (5). However, it is possible that the six residue kinase site placed onto the C-terminus interferes with DNA binding of EBNA1^{PK}. Hence, we first performed experiments to show that the EBNA1^{PK} interacts with the dyad symmetry element of *oriP*, and measured the stoichiometry of this interaction.

The analysis of EBNA1^{PK} bound to the dyad symmetry element is shown by two different methods in Figure 3. In Figure 3A, EBNA1^{PK} was titrated into reactions containing an end-labeled DNA fragment containing the dyad symmetry element and then the reactions were analyzed in a native polyacrylamide gel. The dyad symmetry element contains four EBNA1 binding sites and the several mobility shifted bands observed during the early phase of the titration may be explained by the gradual filling of the several sites. At levels of 560 fmol of EBNA1^{PK} and above, all the DNA was present in the band exhibiting the greatest mobility shift (although not shown in the figure, <5 % of total radioactivity resided in the gel well). Hence, saturation is achieved between 2.8 and 5.6 dimers of EBNA1^{PK} added to each dyad symmetry element. The observed range of EBNA1^{PK} needed to saturate the DNA fragment is consistent with the four EBNA1 binding sites contained within the dyad symmetry element of *oriP*. For a DNA fragment containing four sites, four gel shifted bands should be observed. The results showed what appear to be five bands. It is possible that bands 2 and 3 are isomers, each containing two EBNA1^{PK} dimers but at different sites in the dyad symmetry element. Alternatively, five EBNA1^{PK} dimers are capable of assembling onto the four site dyad. In summary these results show that EBNA1^{PK} is capable

A) Gel Mobility Shift

B) Gel Filtration

Figure 3. EBNA1^{PK} binds the dyad symmetry element of *oriP*. Interaction between EBNA1^{PK} and the dyad symmetry element was examined by two methods. (A) EBNA1^{PK} was titrated from 0 to 1120 fmol (as dimer) into a reaction containing 100 fmol of an end-labeled DNA fragment containing the dyad symmetry element. Reactions were then analyzed for protein–DNA complexes detected as mobility shifted bands in a native polyacrylamide gel. The end-labeled DNA is labeled ‘Dyad DNA’ to the left of the gel. The five gel shifted bands are also indicated to the left. The amount of EBNA1^{PK} added to each reaction is shown above the gel. (B) Top; a plasmid containing the dyad symmetry element of *oriP* (pGEMdyad) was mixed with an excess of [³²P]EBNA1^{PK} (3-fold excess of [³²P]EBNA1^{PK} over binding sites). The reaction was then gel filtered and to resolve protein–DNA complexes (indicated by the first arrow at the top) from free protein (indicated by the second arrow). Fractions were quantitated for [³²P]EBNA1^{PK} (circles) and pGEMdyad (squares). Bottom; as a negative control for non-specific binding of [³²P]-EBNA1^{PK} to DNA, the experiment was repeated using pGEM II which lacks the dyad symmetry element.

of binding the dyad symmetry element, and that they do so in a range that is consistent with the number of EBNA1 sites in the dyad symmetry element.

In Figure 3B (top) the stoichiometry of EBNA1^{PK} bound to the dyad symmetry element was measured using a gel filtration approach. In this experiment [³²P]EBNA1^{PK} was added to pGEMdyad, a plasmid containing the dyad symmetry element of *oriP*. The [³²P]EBNA1^{PK} was present at an 11-fold excess over the plasmid (2.8-fold excess over the DNA binding sites in pGEMdyad) to ensure saturation of the dyad with [³²P]EBNA1^{PK}. The reaction was then gel filtered over a large pore resin that excludes large protein–DNA complexes (they elute in the void fractions which resolve from free protein that elute later, in the included volume). The fractions were analyzed for the amount of [³²P]EBNA1^{PK} (from the known specific activity) and for the amount of DNA (by absorbance at 260 nm). The results, in Figure 3 (top), show comigration of [³²P]EBNA1^{PK} with the pGEMdyad plasmid in the excluded fractions (fractions 10–13) and unbound [³²P]EBNA1^{PK} in the included fractions (fractions 17–40). As a control for non-specific binding to DNA, this reaction was repeated using pGEM II (Fig. 3, bottom). The result showed very little non-specific binding of [³²P]EBNA1^{PK} to DNA.

Table 1. Stoichiometry of interaction between [³²P]EBNA1^{PK} and pGEM dyad

Fraction	[³² P]EBNA1 ^{PK} (pmol)	pGEMdyad (pmol)	[³² P]EBNA1 ^{PK} –pGEMdyad (pmol)
10	5.7	1.1	5.2
11	11.2	2.5	4.5
12	6.8	1.3	5.2
13	3.3	0.6	5.5
		Average:	5.1

The column fractions containing [³²P]EBNA1^{PK}–pGEMdyad complex were quantitated for pmol content of DNA and [³²P]EBNA1^{PK} as described in Materials and Methods. The column fractions are those of the experiment in Figure 3 in which [³²P]EBNA1^{PK} was incubated with pGEMdyad and then gel filtered to remove free [³²P]EBNA1^{PK}.

The quantitation of [³²P]EBNA1^{PK} and pGEMdyad plasmid in the excluded fractions is shown in Table 1. The analysis gives an average value of 5.1 [³²P]EBNA1^{PK} dimers bound to each pGEMdyad plasmid. Although the dyad symmetry element has only four EBNA1 binding sites, it is possible that it binds five EBNA1 dimers; four dimers to the four sites and one dimer

Figure 4. EBNA1 interacts with the 70 kDa subunit of RPA. Interaction of bEBNA1 with RPA and its subassemblies was analyzed by surface plasmon resonance. Immobilized on a sensor chip using carbodiimide chemistry were: (A) recombinant RPA (1853 RU final), (B) 32•14^{his} kDa complex (1510 RU final) and (C) 70^{his} kDa subunit (1080 RU final). Solutions of bEBNA1 were passed over immobilized proteins for 6 min followed by buffer lacking protein. The positions of protein injection and buffer injection are marked by arrows.

through protein-protein interaction. Five dimers of [³²P]EBNA1^{PK} bound to each dyad symmetry element is also consistent with the five mobility shifted bands observed in Figure 3A. However, despite these measurements, inaccuracies in the methods do not exclude the possibility that the stoichiometry is only four EBNA1 dimers bound to one dyad symmetry element.

The experiments of Figure 3 showed that EBNA1^{PK} was capable of binding to the dyad symmetry element of *oriP*, and identified the approximate stoichiometry of EBNA1^{PK} on the dyad symmetry element. This information allowed us to design a kinase protection assay to determine whether RPA could associate with EBNA1^{PK} while bound to the dyad symmetry element. In Figure 2B, a ratio of two EBNA1^{PK} dimers to one dyad symmetry element was used to ensure full binding of EBNA1^{PK} to the four site dyad symmetry DNA. The result showed that in the presence of RPA, phosphorylation of EBNA1^{PK} was inhibited >50% (the β^{PK} internal control was not inhibited by DNA). Hence, this experiment shows that RPA not only interacts with EBNA1 in solution, but also interacts with EBNA1 when it is bound to origin DNA.

The 70 kDa subunit of RPA interacts with EBNA1

The trimeric RPA, and a heterodimer of the 32•14^{his} kDa subunits, are expressed as soluble proteins in *E.coli* (36). Soluble trimer and 32•14 kDa heterodimer are also expressed in the baculovirus system (40). This made possible the test of whether RPA, lacking the 70 kDa subunit, could interact with EBNA1. This was examined in Figure 4 using surface plasmon resonance. Recombinant RPA and 32•14^{his} kDa complex were attached to sensor chips. In the experiments of Figures 1 and 2 the EBNA1^{PK} was utilized for the purpose of examining the interaction of RPA with EBNA1 in two different assays. For the experiment of Figure 4, we utilized a derivative of EBNA1 produced in baculovirus, bEBNA1, which lacks only six N-terminal residues and most of the non-essential Gly-Ala repeat region (17). Hence, bEBNA1 contains ~225 N-terminal residues that EBNA1^{PK} lacks.

Figure 4A shows the interaction of bEBNA1 with immobilized recombinant RPA. A stronger interaction was observed than using EBNA1^{PK}, indicating that other areas of EBNA1, lacking in EBNA1^{PK}, also contribute to its interaction with RPA. Consistent with this result, ELISA experiments have identified two regions of EBNA1 that interact with RPA; one encompassing the C-terminus and a second spanning the internal portion of EBNA1, N-terminal to EBNA1^{PK} (Weisschart, K., Frappier, L. and Fanning, E., submitted). Figure 4B shows the results of passing bEBNA1 over the immobilized 32•14^{his} kDa complex. Only a slight amount of interaction was observed between the 32•14^{his} kDa complex and bEBNA1, and when the injection ended, the signal dropped rapidly to baseline. The slight interaction between bEBNA1 and the 32•14^{his} kDa complex suggests that the major contact of RPA to EBNA1 may be mediated by the 70 kDa subunit of RPA. The 70 kDa subunit is insoluble when expressed alone in *E.coli* (33,36). Thus, to directly test the interaction between RPA and the 70 kDa subunit, the 70^{his} kDa subunit was purified in urea and then renatured. The soluble renatured 70^{his} kDa subunit was immobilized on a sensor chip and bEBNA1 was passed over it. As predicted, an interaction with bEBNA1 was observed upon passing bEBNA1 over the immobilized 70^{his} kDa subunit (Fig. 4C). Similar results were obtained with a soluble fragment of the 70 kDa subunit of RPA in an ELISA assay (Weisschart, K., Frappier, L. and Fanning, E., submitted). The RU observed for interaction between bEBNA1 and 70 kDa alone should be ~60% that observed for intact RPA (116 kDa/70 kDa = 60%), yet the final RU observed using 100 nM 70 kDa subunit is ~10% that observed using 100 nM RPA (compare Fig. 4A and C). It is possible that the decreased efficiency of interaction using 70 kDa subunit relative to intact RPA is due to the denaturation-renaturation procedure used to prepare the 70 kDa subunit. This may have resulted in a significant population of inactive 70 kDa subunit in the preparation. Alternatively, the 32•14 kDa complex may contribute to interaction with bEBNA1 in the context of the intact RPA complex.

What is the function of the RPA-EBNA1 interaction?

Four dimers of EBNA1 interact with the four EBNA1 binding sites within the dyad symmetry element of *oriP* (17), and the DNA appears wrapped around the protein in electron microscopic observations (9,10). Studies using potassium permanganate show that EBNA1 induces structural distortions at the dyad symmetry element of *oriP* (14–16). The experiments described here show

Figure 5. Putative model of events at the dyad symmetry element of *oriP*. The 70 kDa subunit of RPA interacts with EBNA1 at the dyad symmetry element of the latent origin (*oriP*) of EBV. The 70 kDa subunit of RPA is the ssDNA binding subunit. RPA interacts with other protein including DNA polymerase α -primase. Hence, RPA may help recruit other proteins to the origin.

that the 70 kDa subunit of RPA interacts with EBNA1, both in solution and when EBNA1 is bound to the origin (diagrammed in Fig. 5). Although we detect no influence of RPA on the EBNA1 induced distortion of the dyad symmetry element (unpublished), RPA may help destabilize the duplex when present in combination with other proteins. As RPA is known to interact with several different proteins, including the DNA polymerase α -primase (25), it seems possible that a functional consequence of RPA assembly into the EBV orisome may be to help recruit host factors to the origin.

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