Regulation of the double-stranded RNA-dependent protein kinase PKR by RNAs encoded by a repeated sequence in the Epstein–Barr virus genome

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ABSTRACT

During the initial infection of B lymphocytes by Epstein–Barr virus (EBV) only a few viral genes are expressed, six of which encode the EBV nuclear antigens, EBNAs 1–6. The majority of EBNAs share common 5′-ends containing a variable number of two alternating and repeated exons transcribed from the BamHI W major internal repeats of the viral DNA. These sequences can also exist as independent small RNA species in some EBV-infected cell types. We present evidence that transcripts from these W repeat regions can exert a trans-acting effect on protein synthesis, through their ability to activate the dsRNA-dependent protein kinase PKR. UV cross-linking and filter binding assays have demonstrated that the W transcripts bind specifically to PKR and can compete with another EBV-encoded small RNA, EBER-1, which was shown previously to bind this kinase. In the reticulocyte lysate system the W RNAs shut off protein synthesis through an ability to activate PKR. In contrast to EBER-1, the W RNAs are unable to block the dsRNA-dependent activation of PKR. Using a purified preparation of the protein kinase we have shown that the W transcripts directly activate PKR in vitro. The results suggest that EBV has the ability both to activate and to inhibit PKR through the actions of different products of viral transcription.

INTRODUCTION

Epstein–Barr virus (EBV) is linked with a number of human malignancies, including Burkitt’s lymphoma, nasopharyngeal carcinoma and lymphoproliferative diseases in immunocompromised patients. In vitro, EBV is able to efficiently immortalise normal human B lymphocytes and in the majority of the infected cells the virus enters into a latent state in which only a few viral genes are expressed (1). These genes can encode up to six nuclear antigens (EBNAs 1–6), three membrane proteins (LMP 1, 2A and 2B) and two small RNAs (EBER-1 and -2) (2). Since the coordinated action of these gene products appears to be sufficient to promote cell proliferation and immortalisation, it is important to understand their biological effects and the mechanisms by which their expression is regulated.

The EBNAs are translated from mRNAs which arise from complex primary transcripts that span a large portion of the viral genome. Two distinct promoters that can direct synthesis of such RNAs have been identified, located in the BamHI C unique region and the BamHI W major internal repeat sequence of the viral genome respectively (3–6). The messages for each individual EBNAs are generated by differential splicing and these RNAs can have a number of variations of exon structure at their 5′-ends (7–9). Additional promoters for the synthesis of transcripts encoding EBER-1 have also been identified in the BamHI F and Q regions of the genome (10–14). With the exception of this last case, all the EBNAs messages contain their 5′-ends multiple copies of a sequence encoded by a pair of exons, W1 (66 nt) and W2 (132 nt), within each BamHI W repeat. These W1W2 exon pairs usually occur within the 5′ untranslated regions (5′ UTRs) of the EBNAs mRNAs, although in the case of EBNAs-5 (EBNA-LP) a different splicing pattern creates an upstream AUG which permits translation of the W repeats (15). The significance of the W repeat sequences in the 5′ UTRs of mRNAs encoding the EBNAs is unclear, but these regions are GC-rich and may adopt stable secondary structures (16). Such features in eukaryotic mRNA are known to reduce the efficiency of translation of downstream open reading frames (17,18).

The significance of transcription of the W repeat region of the EBV genome is further complicated by the evidence for the presence of small RNA species containing W exons in cell lines latently infected with the virus (16,19). These small RNAs are of unknown function, but may act in trans to regulate gene expression at the post-transcriptional level.

The interferon-inducible protein kinase PKR (also known as DAI and p68) is intimately involved in the regulation of protein synthesis in response to viral infections (20,21). This enzyme is dependent for its activation on double-stranded RNA (dsRNA), which can be produced by symmetrical transcription of viral genomes. Extensive stem–loop structures in mRNAs may also activate PKR (22,23). The computer-predicted secondary structure

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of the W1W2 exon pair transcribed from the BamHI W repeat sequence of EBV indicates such a possible hairpin structure (Fig. 1). Indeed, limited RNase cleavage of end-labelled W1W2 transcripts confirms that this RNA adopts a complex secondary structure (data not shown). These data suggested to us that this RNA sequence may have the ability to bind to PKR and activate the protein kinase. Moreover, the presence of the W-encoded RNA sequence at the 5′-ends of the EBNA mRNAs is reminiscent of the situation with HIV-1 RNAs, where the TAR sequence is a common 5′-element (24). TAR has been shown to bind to PKR and regulate the activity of the enzyme, although there has been some controversy over whether PKR is activated or inhibited by TAR (25–29).

We have investigated the possibility that the EBV BamHI W-encoded RNA may be a regulator of PKR activity. In the study reported here we describe evidence for a functional interaction between this RNA and PKR using a variety of binding experiments, in vitro protein synthesis measurements and autophosphorylation assays.

**MATERIALS AND METHODS**

**PKR**

HeLa cells were grown in spinner culture in RPMI 1640 (Gibco) supplemented with 10% heat-inactivated fetal calf serum (Imperial Laboratories) and diluted every two or three days to between 1.5 and $4 \times 10^5$ cells/ml. Wild-type PKR was purified from a ribosomal salt wash obtained from 40 l of cells that had been treated with human interferon α (1000 U/ml for 24 h) (30). The protein was subjected to chromatography on DEAE–cellulose (where it elutes in the flow-through fraction), followed by chromatography on phosphocellulose. Proteins were eluted with a 50–500 mM KCl gradient and the PKR-containing fractions (eluting at ∼250 mM KCl) were identified by Western blotting. All buffers used during purification contained aprotinin (1 µg/ml), leupeptin (1 µg/ml), pepstatin (1 µg/ml) and PMSF (1 µM). The K296R mutant of PKR was purified from insect cells infected with a recombinant baculovirus expressing this protein as described previously (31,32).

**Plasmids**

Plasmids containing various numbers of copies of the W1W2 exon pair, cloned in the pSP64 transcription vector, were kindly provided by Prof. Paul Farrell (Ludwig Institute for Cancer Research, London). These were modified to remove a region of the multiple cloning site lying between the SP6 RNA polymerase promoter and the W1W2 sequences. Plasmids containing one, two or seven W1W2 repeats were generated and shown to be suitable for transcription of the corresponding RNAs using SP6 polymerase. Plasmids encoding the EBV small RNA EBER-1, derived from that described by Clarke et al. (33), and a Xenopus laevis tRNA^Phe^ precursor (a gift from Dr G. Pruijn, University of Nijmegen, The Netherlands) were transcribed with T7 RNA polymerase as previously described (34).

**Other materials**

SP6 and T7 RNA polymerases were purchased from Cambio and poly(dI)-poly(dC) and 2-aminopurine from Sigma Chemical Co. Radiochemicals were from ICN Flow or Amersham International. Reticulocyte lysates were a kind gift from Drs Simon Morley and Jenny Pain (University of Sussex, Falmer, UK).

**In vitro transcription and RNA purification**

Small RNAs were synthesised by in vitro transcription from recombinant plasmids using either SP6 or T7 RNA polymerases (35). Plasmids were linearised with the appropriate restriction enzymes such that there would be no extraneous sequences at the 3′-ends of the transcripts and the templates were gel purified before use. Transcription was performed in a final volume of 100 µl for 3 h at 37°C. The DNA template was removed by
adding 15 U DNase I at 37°C for 15 min and the RNA was phenol extracted and precipitated with isopropanol.

Radiolabelled transcripts were produced using a similar protocol except that unlabelled UTP was omitted from the reaction and replaced with 50 μCi [α-32P]UTP. Labelled RNA was quantified by precipitating 1–2 μl of a dilution of the transcript with cold 10% trichloroacetic acid, 0.5% sodium pyrophosphate, followed by scintillation counting.

RNA preparations synthesised in vitro often include a minor population of dsRNA contaminants (33,36). In experiments involving assays of PKR activation it is therefore often necessary to purify transcripts to remove such contaminants and this was carried out as described by Mellits et al. (36). Approximately 150–300 μg RNA were loaded onto a 5% denaturing polyacrylamide gel using formamide loading buffer as previously described. After electrophoresis the RNA was detected by UV shadowing, eluted for 3 h at 37°C into buffer (0.5 M NaCl, 5 mM EDTA, 80 mM HEPES, pH 7.6) containing 1200 U/ml RNAguard (Pharmacia) and ethanol precipitated with 20 μg glycogen as carrier. The eluted RNA was then run on a 5% non-denaturing polyacrylamide gel and recovered using the same procedure as before.

Assays for RNA–protein interactions

UV cross-linking of RNA samples to purified PKR was carried out as described previously (37). For convenience the K296R mutant form of PKR was used in some experiments, as this protein can be easily produced from insect cells infected with a recombinant baculovirus (31,32). Although this mutant has lost all protein kinase activity it retains the RNA binding characteristics of the wild-type enzyme (reviewed in 21). 32P-Labelled RNA (10⁵ c.p.m.) was incubated with PKR preparations for 15 min at 30°C in the presence of 80–100 mM KCl, 10 mM Tris–HCl, pH 7.5, in a final volume of 25 μl. RNA was cross-linked to protein by irradiation at 254 nm for 5 min at 4°C. The samples were then made 0.5% in N-laurylsarcosine. Each sample was treated with 20 U RNase T₁ and 20 μg RNase A for 1 h at 37°C and analysed by SDS gel electrophoresis. Cross-linked complexes were identified by autoradiography.

The formation of RNA–protein complexes was also assayed by retention of radioactivity on cellulose nitrate filters. Varying concentrations of RNA were incubated with mutant PKR in the presence of 10 mM Tris, pH 7.6, 100–150 mM KCl and 0.8 mM Mg acetate for 15 min as described previously (34). Labelled RNAs were present at 10⁵–10⁶ c.p.m. When competition assays were performed a second non-radioactive RNA was also included at increasing molar excess over the labelled species.

Protein synthesis assays

Endogenous protein synthesis was measured in rabbit reticulocyte lysates by the incorporation of l-[3H]leucine into acid-insoluble material, in the presence or absence of the synthetic dsRNA poly(I)poly(C) (0.1 μg/ml) (33,38). Reactions were incubated at 30°C for 1 h and triplicate aliquots removed at the end of the incubation. Hot trichloroacetic acid-insoluble radioactivity was measured by liquid scintillation counting. Where indicated the PKR inhibitor 2-aminopurine was included at a concentration of 10 mM.

Measurement of PKR activation

Activation of PKR was assessed by autophosphorylation of the protein kinase in the presence of [γ-32P]ATP. Incubations (20 μl) contained: 10 mM Tris–HCl, pH 7.5, 100 mM KCl, 2 mM MnCl₂, 0.1 mM EDTA, 20% (v/v) glycerol and 4.5 μM [γ-32P]ATP (5–10 μCi). Poly(I)-poly(C) or other RNA species were added as indicated in the figure legends. After 20 min at 30°C proteins were denatured with SDS gel sample buffer and separated by electrophoresis on 15% polyacrylamide–SDS gels. The dried gels were analysed by autoradiography.

RESULTS

Binding of RNAs containing W repeats to the protein kinase PKR

Since W exon transcripts are widely expressed in cells after infection with EBV and are present at the 5′-ends of several EBNA-encoding mRNAs, as well as existing as small cytoplasmic RNA species (16,19), we have investigated the possibility that these W sequences may have a trans-acting function in the cytoplasm of EBV-infected cells. Structural analysis of the W1W2 RNA suggests that this GC-rich sequence has extensive secondary structure (Fig. 1 and data not shown). We therefore examined whether the small W RNAs can interact with the dsRNA-dependent protein kinase PKR. This enzyme shows specific and tight binding of a number of small viral RNAs, including V&A RNA of adenovirus (27,39,40), the TAR RNA of HIV-1 (26,28) and EBER-1 and EBER-2 of EBV (34,37).

To assess their ability to bind to PKR, RNA species containing one, two and seven copies of the 198 nt W1W2 repeat sequence were synthesised by in vitro transcription using SP6 RNA polymerase. For direct binding studies each small RNA was labelled by transcription in the presence of [α-32P]UTP, incubated with purified preparations of wild-type or K296R mutant PKR and subjected to UV cross-linking as described in Materials and Methods. Figure 2 shows that, on analysis by SDS gel electrophoresis, all three W1W2 RNAs were found to be cross-linked to both forms of the 68 kDa PKR protein. The (W1W2)₇ RNA gave a somewhat stronger signal than the W1W2 and (W1W2)₂ species. As reported previously (34,37), the EBV-encoded small RNA EBER-1 could also be cross-linked to wild-type PKR and the K296R mutant (although cross-linking to the wild-type is weak in Fig. 2A), but a RNA transcript could not be cross-linked (Fig. 2C). These data indicate that, like the other small viral RNAs described above, the W1W2 RNAs can bind to PKR in vitro. As has been clearly established in the case of other ligands, the protein kinase activity of PKR is not required for RNA binding.

We have also assayed binding of the small RNAs to PKR by a filter binding method. Figure 3 shows that W1W2 RNA bound to increasing concentrations of purified recombinant PKR with a similar concentration curve to that shown by EBER-1. In both cases binding increased up to a PKR concentration of ~10 μg/ml in the incubation; above this concentration little further RNA binding was observed, probably due to PKR being in excess over the ligand or to saturation of the filters with protein. Using such an assay we then examined whether the two RNA sequences compete with each other for binding to PKR. As shown in Figure 4, the binding of labelled W1W2 RNA to PKR was competed out in a similar manner by both unlabelled EBER-1 and
UV cross-linking of small RNAs to PKR. RNAs containing one, two or seven W1W2 repeats were labelled by in vitro transcription in the presence of [α-32P]UTP. EBV-encoded EBER-1 and a Xenopus laevis tRNA Phe precursor (56) were also synthesized in parallel, for use as positive and negative controls respectively. The labelled small RNAs (10^5 c.p.m.) were incubated with either wild-type PKR (200 µg/ml), purified from HeLa cells (A), or the mutant PKR K296R (140 µg/ml), purified from recombinant baculovirus-infected insect cells (B and C). The complexes were subjected to UV cross-linking and RNase digestion and then analysed by SDS gel electrophoresis followed by autoradiography. (A and B) Lane 1, labelled protein molecular weight markers; lane 2, EBER-1; lane 3, W1W2 one repeat RNA; lane 4, (W1W2)2 RNA; lane 5, (W1W2)7 RNA. (C) Lane 1, labelled protein molecular weight markers; lane 2, tRNA; lane 3, EBER-1. The arrows show the position of the PKR band (68 kDa).

Filter binding assay for association of W1W2 RNA and EBER-1 with PKR in vitro. W1W2 one repeat RNA and EBER-1 were labelled by in vitro transcription as described in Materials and Methods. Each RNA (2.5 × 10^4 c.p.m.) was incubated with increasing concentrations of recombinant PKR K296R in a volume of 25 µl. Formation of RNA–protein complexes was quantified by retention of radioactivity on cellulose nitrate filters, followed by scintillation counting (34). Note the logarithmic scale for PKR concentration.

(W1W2)2 RNA, as well as by the unlabelled W1W2 RNA itself. In contrast, unlabelled tRNA was a poor competitor when the binding of each of these RNAs to PKR was assayed; only a 1000-fold molar excess of tRNA had any significant effect on binding. These results suggest that binding of the W repeat RNAs to PKR is specific.

Effects of W repeat RNAs on PKR activity and protein synthesis

We next investigated whether the binding of the W repeat RNAs to PKR has any functional significance for the activity of the protein kinase. When activated by dsRNA PKR undergoes autophosphorylation and then phosphorylates the α subunit of protein synthesis initiation factor eIF2 at position Ser51 (reviewed in 21). This inhibits polypeptide chain initiation by blocking the activity of the guanine nucleotide exchange factor eIF2B (41). The reticulocyte lysate cell-free translation system contains endogenous PKR and is a suitable assay system for examining the effects on protein synthesis of RNA molecules that regulate this protein kinase (28,33). The effects of gel-purified W1W2 RNA in this system were assayed in the presence and absence of the synthetic PKR activator poly(I)-poly(C) (Fig. 5A). In the absence of poly(I)-poly(C) the W RNA caused a marked shut-off of protein synthesis. The maximum level of inhibition was identical to that caused by poly(I)-poly(C) itself, with 50% of maximum being achieved at a concentration of between 0.1 and 1 µg/ml (although this was somewhat variable between experiments; compare Fig. 5A and B). In the presence of poly(I)-poly(C) at a concentration sufficient for maximal activation of PKR the W RNA had no further effect on overall protein synthesis, suggesting that even at concentrations as high as 100 µg/ml the W RNA is unable to block activation of PKR by dsRNA. Inhibition of protein synthesis in the reticulocyte lysate by the W1W2 RNA, like that caused by poly(I)-poly(C), was reversed by the PKR inhibitor 2-aminopurine (Fig. 5B). This suggests that the effect of the W RNA is mediated by PKR activation rather than being a consequence of a non-specific inhibition of translation.

The ability of RNAs containing different numbers of W1W2 exon repeats to directly activate PKR was assayed by examining the autophosphorylation of the purified protein kinase, using a wide range of RNA concentrations (Fig. 6). Poly(I)-poly(C) was used in this assay as a positive control. The latter stimulated autophosphorylation of PKR at concentrations as low as 0.01 µg/ml and was maximally active at 0.1–1 µg/ml (Fig. 6A). Higher concentrations of poly(I)-poly(C) were inhibitory, as observed in several previous studies (42–44). In confirmation of the effects on protein synthesis noted earlier, the W RNAs also induced autophosphorylation of PKR at concentrations as low as 0.01 µg/ml and was maximally active at 0.1–1 µg/ml (Fig. 6A). Higher concentrations of poly(I)-poly(C) were inhibitory, as observed in several previous studies (42–44). In confirmation of the effects on protein synthesis noted earlier, the W RNAs also induced autophosphorylation of PKR, although ∼10-fold higher concentrations of the RNAs were required. When compared on an equimolar basis, (W1W2)2 RNA was more effective than (W1W2)2 RNA, which in turn was more potent than W1W2 one repeat RNA as an activator of PKR autophosphorylation (Fig. 6B).
Origin and significance of the W repeat RNAs

Several studies have demonstrated the complexity of the patterns of transcription and splicing of the BamHI W internal repeats during EBV infection (7–9). Although the W exons are present close to the 5′-ends of mRNAs for all the EBNA proteins, except in some situations EBNA-1 (10,11), in the majority of cases they are not translated into protein but constitute a major part of the 5′ UTRs of these mRNAs. However, in the case of EBNA-5 (EBNA-LP) the W1W2 exon repeats code for a repeated amino acid sequence, as a consequence of the creation of an upstream AUG by a different splicing event (15).

Previous evidence from our laboratory suggested that, in addition to the presence of the W exons in the EBNA mRNAs, these sequences could be found in a family of small RNAs expressed in relatively larger amounts in the cytoplasm of EBV-infected cells (16). The exact nature of these small W RNAs remains to be established, but the presence of several species with size differences which are multiples of ∼200 nt (16) suggested that they may contain multiple numbers of W1 and W2 exon pairs (consisting of 198 nt/repeat; 8). Alfieri et al. (19) also detected a 200 nt RNA species which was present in both acutely EBV-infected lymphocytes and latently infected IB-4 cells and which hybridized to an EBNA-5 probe containing W repeat sequences. The molecular basis for the appearance of these small RNAs is not clear, but the phenomenon may reflect the tendency for RNA polymerase II to terminate transcription prematurely when RNA sequences with extensive stable secondary structure are formed (46,47). The existence of the small W RNAs is reminiscent of the situation with the TAR RNA sequence of HIV-1. This highly structured RNA is present at the 5′-ends of all HIV mRNAs, but also exists independently in infected cells as a result of premature termination of viral transcription (46,47). Interestingly, because of its stable hairpin loop structure, TAR RNA also binds to PKR and has been reported to be able to activate the kinase (25,29).

Activation of PKR by W repeat RNAs

Evidence from both in vitro translation experiments and direct assays of PKR autophosphorylation indicates that the W RNAs have the potential to activate the protein kinase. Such activation appears to be a true property of these RNA species and cannot be attributed to the presence of artefactual dsRNA contaminants in the W repeat transcripts.

DISCUSSION

In this paper we have shown that transcripts encoded by the BamHI W region of the major internal repeat (IR1) of the EBV genome bind to the interferon-inducible protein kinase PKR and induce autophosphorylation and activation of the enzyme. Binding to both wild-type PKR and a recombinant mutant form of the protein which is catalytically inactive has been demonstrated. Competition experiments using other PKR ligands and RNA species that do not bind to the protein kinase have demonstrated that the W repeat RNAs bind in a specific manner. The data suggest that this binding may occur at the same site(s) that bind(s) dsRNA activators and other RNA ligands, i.e. the dsRNA binding domains near the N-terminus of the protein (40,45), although this has not been tested directly. Two types of assay, namely autophosphorylation of purified wild-type PKR and inhibition of protein synthesis in the reticulocyte lysate, have shown that gel-purified W transcripts can function as activators of the RNA-dependent protein kinase.

Although gel purification efficiently eliminated dsRNA contaminants from a RNA transcript prepared in a similar way (data not shown), this procedure had relatively little effect on the ability of W RNA to activate the protein kinase. Thus it is unlikely that the effects observed in these experiments were due to any spurious dsRNA contaminants in the W repeat transcripts.
Nevertheless, it is rather surprising that the one repeat W RNA is able to activate PKR because it does not possess any long tracts of uninterrupted base pairing (data not shown). A similar situation appears to exist in the case of nt 416–575 of reovirus S1 mRNA, which is a potent PKR activator (22). It seems likely that additional aspects of the structure of such small RNAs, such as tertiary interactions, are important in the regulation of PKR activity. A single repeat W RNA is also probably too small to bind more than one molecule of PKR at a time, in which case it might be expected that dimerization-dependent activation (21) would not occur. The fact that this RNA can indeed activate the enzyme suggests that RNA-mediated dimerization is not a prerequisite or that only one monomer in a PKR dimer actually needs to be bound to the RNA for activation to take place. However, it is notable that a seven-repeat W transcript is significantly better on a molar basis in promoting PKR autophosphorylation (Fig. 6). Thus larger numbers of the repeat sequence linked in tandem, as occurs in vivo in EBNA mRNAs, may be more effective because they can bind greater numbers of PKR monomers and thus facilitate trans-phosphorylation reactions.

The competition binding studies using W RNAs and EBER-1 indicate that these species may bind to the same (or at least overlapping) sites on PKR. By means of filter binding we have previously measured the $K_d$ for the association of EBER-1 with PKR as being $\sim 0.3$ nM (34), similar to the value estimated by others for dsRNA binding to PKR (40,49) and other proteins (50) using different methods. It would seem likely that the W RNAs bind to PKR with a comparable affinity to that of EBER-1 and other structured RNAs (note that $0.3$ nM for (W1W2)$_7$ RNA is equivalent to a concentration of 0.14 µg/ml). Similar concentrations of the W RNAs and EBER-1 (0.1–1 µg/ml and above) are required to activate and inhibit PKR respectively (this paper and 34). Unlike EBER-1, the W RNAs are not inhibitory towards the dsRNA-dependent activation of the protein kinase, even at very high concentrations.

Implications for the physiology of Epstein–Barr virus infections

The importance of the activation of PKR by transcripts from the major internal repeat region of the viral genome is not yet known, but it may be advantageous to the virus to temporarily shut down host protein synthesis during the early stages of infection. Such an effect has been seen in cells infected with a number of other (predominantly lytic) viruses (51–53) and is probably necessary to allow establishment of infections and the accumulation of early viral mRNAs and proteins. EBNA mRNAs containing W repeat sequences appear at relatively early times after EBV infection of B lymphocytes (19,54).

Later during infection, in spite of the potential activation of PKR by the W RNAs, protein synthesis must be stimulated in order to support B cell proliferation. In this connection it may be significant that the PKR inhibitor EBER-1 appears relatively late in the time course of infection (19), perhaps at a time when the activity of PKR needs to be down-regulated. Since PKR is an interferon-induced protein it may also be pertinent that B cell
infection by EBV is sensitive to inhibition by interferons at early but not at late times after exposure of the cells to the virus (55). The mechanisms responsible for this time-dependent phenomenon have not been elucidated, however, and there is no evidence for or against a role for PKR in the inhibition of EBV infection by interferons. Future studies of the extent of activation of PKR at different times after infection, as well as in established EBV-transformed cell lines, should help to determine whether the ability of EBV to encode RNAs capable of both activating and inhibiting PKR constitutes a means of fine tuning the activity of this protein kinase at different stages in the viral life cycle.

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