Binding of Epstein-Barr virus small RNA EBER-1 to the double-stranded RNA-activated protein kinase DAI

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ABSTRACT

Epstein-Barr virus encodes two small RNAs, EBER-1 and -2, that are abundantly expressed in latently infected cells. Recent evidence suggests a role for EBER-1 in regulation of translation since this RNA is able to prevent the inhibition of protein synthesis by double-stranded RNA in rabbit reticulocyte lysates. We show here that EBER-1 that has been synthesized *in vitro* forms a complex with the dsRNA-activated inhibitor of protein synthesis DAI, a protein kinase that specifically phosphorylates polypeptide chain initiation factor eIF-2. Gel retardation assays and UV crosslinking experiments indicate that complex formation is specific for EBER-1 and requires the presence of some secondary structure in the molecule. RNA competition studies show that EBER-1-DAI complex formation is not inhibited in the presence of other small RNA species, heparin or the synthetic double-stranded RNA, poly(I).poly(C). SDS gel analysis reveals the existence of two forms of the crosslinked complex, of 64 – 68kDa and 46 – 53kDa, both of which are recognized by anti-DAI antibodies in immunoprecipitation experiments. These data suggest that EBER-1 regulates protein synthesis through its ability to interact with DAI.

INTRODUCTION

Epstein-Barr virus, a ubiquitous human herpesvirus, is a causative agent of infectious mononucleosis and lymphoproliferative diseases in immunocompromised hosts and has also been aetiologically associated with endemic Burkitt's lymphoma and nasopharyngeal carcinoma (1). *In vitro* the virus will efficiently immortalise human B lymphocytes, giving rise to autonomously replicating lymphoblastoid cell lines in which the viral genome is maintained as a circular episome in the nuclei of infected cells (2–4). In the majority of infected cells the virus does not replicate but enters a latent state in which expression of the genome is restricted to the genes encoding six nuclear antigens, EBNAs-1, 2, 3a, 3b, LP, and 3c (also termed EBNAs 1–6 respectively), and two membrane proteins (LMP-1 and LMP-2) (5,6). In addition two small untranslated, non-polyadenylated RNAs (EBER-1 and EBER-2) are expressed and accumulate to high levels early in infection (7–10).

EBER-1 and EBER-2 are RNA polymerase III transcripts of 167 and 172 nucleotides respectively which were originally detected in total cytoplasmic and polysomal RNA from EBV-infected cells (11). These RNAs have considerable secondary structure consisting of a number of stable stem-loops (12). Their 3′ U-rich termini have been shown to bind the 46.7kDa La (SS-B) antigen (7), a protein which associates transiently with all RNA polymerase III transcripts in mammalian cells (13).

The physiological function of the EBERs is largely unknown. *In situ* hybridisation data have suggested that these RNAs are localised in the nuclear compartment, perhaps implying a role in RNA processing or transport (14). However, there is also evidence, at least in the case of EBER-1, for a role in the regulation of translation. The EBERs can functionally substitute for an analogous RNA (VA_I RNA) that is expressed at high levels in adenovirus infected cells (15–17). VA_I RNA is known to be involved in preventing the phosphorylation and activation of the interferon-induced 62kDa protein kinase known as the double-stranded RNA-activated inhibitor of protein synthesis (DAI) (18–25). Once activated by double-stranded RNA (dsRNA), DAI phosphorylates the α subunit of protein synthesis initiation factor eIF-2 causing an inhibition of translation at the level of initiation (26,27). We have recently shown that EBER-1 RNA transcribed *in vitro* from a bacteriophage promoter can prevent the dsRNA-mediated inhibition of protein synthesis initiation in reticulocyte lysates (28). These data raised the question of whether EBER-1 can bind to DAI, preventing activation of the protein kinase in a manner analogous to that of VA_I RNA. In the present study we have examined this possibility by performing direct binding studies of EBER-1 with fractions from reticulocyte lysate. RNA-protein interactions were analysed by gel retardation assays, UV-crosslinking studies and immunoprecipitation of crosslinked EBER-1-protein complexes. The data show that EBER-1 does bind to DAI *in vitro* and competition experiments suggest that this binding is specific.

MATERIALS AND METHODS

Synthesis of EBER-1 and other small RNAs

EBER-1 was synthesized *in vitro* by transcription of the plasmid pPAC-1 with T7 RNA polymerase as described elsewhere (28). The RNA was labelled where indicated by including [α^{32}P]UTP

(5 mCi/ml; 3000 Ci/mmol) in the transcription mix. Inosine-substituted EBER-1 was made by replacing GTP with ITP in the transcription mix (28).

The small human cellular cytoplasmic RNAs Y_1 and Y_5 (29) were transcribed from plasmids pLY_1 and pLY_5 respectively using T7 RNA polymerase. A 142 nucleotide fragment containing the 5′ end of rabbit β globin mRNA was transcribed from a plasmid using SP6 RNA polymerase under standard conditions (30).

Preparation of protein fractions

Reticulocyte lysate was prepared according to Jackson and Hunt (31). All subsequent steps were carried out at 4°C. Ribosomes were obtained by centrifugation for 4 h at 200,000×g and the pellet was rinsed twice with buffer C (10 mM Tris-HCl pH 7.6, 250 mM sucrose, 1 mM DTT, 5 mM $Mg(OAc)_2$, 0.25 mM EDTA, 0.2 mM PMSF and 100 U/ml aprotinin) and suspended in this buffer to 350 A_{260}/ml. The ribosomal suspension was brought to 0.5M KCl and after 30 min of stirring the ribosomes were removed by sedimentation for 1 h at 460,000×g. The supernatant was precipitated with ammonium sulphate (0–40%, 40–55% and 55–70% saturation) and the three fractions (SW40, SW55 and SW70) were either dialysed against eIF-buffer (20mM Tris-HCl pH 7.6, 10mM 2-mercaptoethanol, 0.1mM EDTA, 10% glycerol, 0.2 mM PMSF, 100 U/ml aprotinin and 50mM KCl) or applied to a Sephadex G25 superfine column equilibrated with eIF-buffer and the flow-through fraction collected.

Preparation of a DAI-enriched fraction was carried out by applying a 0–55% ammonium sulphate cut of ribosomal salt wash to a DEAE-cellulose column (5mg of protein per ml of column volume) equlibrated with eIF-buffer. The flow-though was collected and the column was then extensively washed with eIF-buffer. A DAI-depleted fraction was eluted with eIF-buffer containing 500mM KCl.

Ribosomal salt washes from control and interferon-treated HeLa cells were prepared by resuspending ribosomes in the minimum possible volume of 20 mM Tris-HCl pH 7.5, 50 mM KCl, 400 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 20% (v/v) glycerol and 1% (v/v) Triton X-100. After 2h at 4°C the ribosomes were centrifuged at 350,000×g for 50 min and the supernatants were collected.

Electrophoretic mobility shift assay

Binding reactions were carried out in 7.5 μl incubations with ^{32}P-labelled EBER-1 RNA (10^4 cpm/ng RNA; $8-10\times10^4$ cpm per reaction) and the respective protein fractions (30 μg protein) in a buffer containing 10 mM Tris-HCl (pH 7.2), 100 mM KCl, 3 mM $Mg(OAc)_2$ and 1 mM EDTA. After incubation at 30°C for 15 min RNases T_1 (1 U/μl) and A (0.1 μg/μl) were added and the incubation was continued for a further 10 min at 30°C. Complexes were analysed on 4% non-denaturing gels and detected by autoradiography as described by Konarska and Sharp (32). Labelled RNA and unlabelled competitors were added simultaneously except where otherwise mentioned. Where indicated treatment with proteinase K (0.1 mg/ml) was carried out following complex formation and RNase digestion, for a further 15 min.

UV-crosslinking

Nuclease treated rabbit reticulocyte lysate or protein fractions (60 μg protein) were incubated for 30 min at 30°C with labelled RNA (ca. 10^4 cpm/ng RNA; 10^5 cpm per reaction). All samples contained 5 mg/ml heparin, 80 mM KCl and 20 mM creatine phosphate, unless indicated otherwise, and had a final volume of 30 μl. Irradiation at 254 nm was performed in pre-cooled 10 cm petri-dishes for 20 min with an 8W germicidal lamp (Steril-Air G-9) at a distance of about 4 cm. Samples were then made 0.5% (w/v) in N-laurylsarcosine. Each sample was digested with 20 units RNase T_1 and 20 μg RNase A for 1h at 37°C. Where indicated proteinase K treatment was carried out by further incubation as described above. 25 μl of 3× electrophoresis sample buffer (240 mM Tris-HCl, pH 6.8, 300 mM dithioerythritol, 30% glycerol, 6% SDS) was added and the samples were subsequently electrophoresed on 10% SDS gels with a constant voltage of about 30–40 V for 15–20 h. Proteins covalently crosslinked to RNA were detected by autoradiography.

Immunoprecipitation of crosslinked complexes

After crosslinking of proteins to RNA 0.2 volumes of 5× immunoprecipitation buffer (0.1 M Tris-HCl, pH 7.8, 0.75 M NaCl, 0.25 M NaF, 25 mM EDTA) were added to each sample. All samples were diluted with 1× immunoprecipitation buffer to a final volume of 300 μl. Subsequently 3 μl of anti-DAI guinea-pig serum or control guinea-pig serum supplemented with 8 mg/ml guinea-pig IgG were added and samples were shaken overnight at 4°C. 10 μl of swollen protein A-agarose was added to each sample and shaking continued for 1h at 4°C. After centrifugation for 2 min in a microfuge the supernatants were removed and the pellets were washed twice with 0.5 ml phosphate buffered saline containing 0.5% Triton X-100 and six times with PBS alone by thoroughly resuspending the pellets with a vortex mixer. 50 μl of 3× electrophoresis sample buffer including 20 units RNase T1 and 2 μg RNase A were added to each pellet followed by incubation of the samples for 1h at 37°C. After heating the samples for 10 min at 90°C. After heating the samples for 10 min at 90°C and centrifugation in a microfuge the supernatants were subjected to SDS-PAGE. The immunoprecipitated complexes were subsequently detected by autoradiography.

Immunoblotting of DAI

After separation by gel electrophoresis proteins were transferred to nitrocellulose membranes and DAI was detected with a monoclonal anti-DAI antibody (33). Antibody binding was quantified with biotinylated sheep anti-mouse immunoglobulin and a streptavidin-alkaline phosphatase conjugate-based detection kit (Amersham International).

RESULTS

Complex formation in ribosomal salt wash fractions

Labelled EBER-1 was initially used in gel retardation experiments to examine complex formation. This assay uses RNA labelled to a high specific radioactivity by *in vitro* transcription. Binding of RNA to protein is indicated by a retardation of the labelled RNA on a non-denaturing polyacrylamide gel, caused by its association with protein (34).

When unfractionated micrococcal nuclease-treated rabbit reticulocyte lysate was used for complex formation, only a faint band was detectable (data not shown). However, when a concentrated 0–40% ammonium sulphate cut of a reticulocyte lysate ribosomal salt wash (SW40) was assayed for EBER-1 binding a pronounced shift in labelled RNA due to the formation of an EBER-1-protein complex was observed (Fig. 1A). Other protein sources were also examined for complex formation. A

single complex of a similar mobility, but giving a weaker signal, was detected using the 40–55% ammonium sulphate cut of a rabbit reticulocyte lysate ribosomal salt wash (SW55); a strong signal was obtained with a fraction from DEAE-cellulose chromatography that was enriched for DAI (Fig 1B). Complexes were not detectable in the 55–70% ammonium sulphate cut of a rabbit reticulocyte lysate ribosomal salt wash (SW70) or the 'DAI-depleted' 500mM KCl eluate from the DEAE-cellulose column described above (Fig. 1B). A single complex was also observed in a supernatant containing native ribosomal subunits remaining after centrifugation of a polysomal preparation from mouse erythroleukaemia cells (not shown), and in a ribosomal salt wash prepared from HeLa cells (Fig. 1C). In the latter case prior treatment of the cells with interferon enhanced the signal in the gel retardation assay. Incubation of the reticulocyte SW40 fraction with proteinase K after complex formation prevented the appearance of a band, confirming the presence of protein in the observed complex (Fig. 1D).

Specificity of complex formation with EBER-1

We have previously described the presence of DAI-activating dsRNA contaminants in EBER-1 transcripts synthesised *in vitro* using T7 bacteriophage RNA polymerase (28). These contaminants are thought to arise from non-specific initiation and transcription of both strands of the template (35). We have shown that in a reticulocyte lysate translation assay RNase T_1 pre-treated transcripts are inhibitory, due to these RNase T_1-insensitive dsRNA contaminants. It was therefore necessary in the present experiments to ensure that complex formation was due to EBER-1-protein interactions and not to contaminating dsRNA-protein interactions.

Labelled EBER-1 was pre-incubated in the presence of either the dsRNA-specific nuclease RNase III or an RNase T_1/RNase A mix prior to complex formation. EBER-1 is not susceptible to RNase III digestion but is digested by the single-strand specific RNase T_1/RNase A mix (Fig 1A), whereas the dsRNA contaminants are sensitive to RNase III digestion but not to RNase T_1/RNase A digestion (28). Fig. 2 shows that complex formation using either the SW40 or DAI-enriched protein fractions is insensitive to RNase III pre-treatment of the labelled EBER-1 but is totally abolished by pre-incubation with the single strand-specific RNases. This sensitivity of complex formation to single strand-specific RNases indicates that complex formation is not due to dsRNA contaminants but is EBER-1 specific.

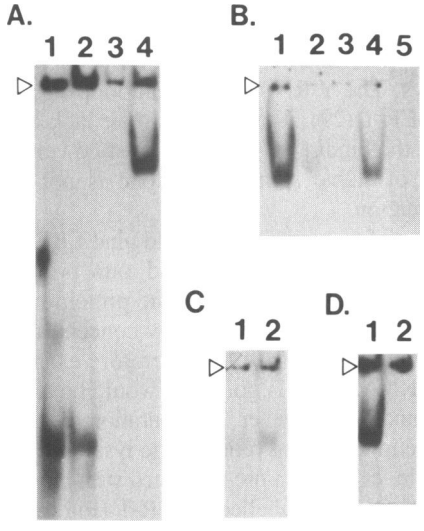

Figure 1. Analysis of EBER-1-protein complexes by gel retardation assays. Binding reactions and gel electrophoresis were carried out as described in Materials and methods. **Panel A** shows the migration of:- **Lane 1**: ^{32}P-EBER-1 alone; **lane 2**: EBER-1 treated with RNase III (2 U/μl at 37°C for 15 min); **lane 3**: EBER-1 treated with RNases T_1 and A (10 U/μl and 10 μg/μl respectively at 30°C for 15 min); **lane 4**: EBER-1 incubated with reticulocyte SW40 salt-wash fraction prior to treatment with RNases T_1 and A. Approximately 10^5 cpm were loaded on each lane of the gel. **Panel B** shows complexes of EBER-1 formed with various reticulocyte fractions:- **Lane 1**: SW40; **lane 2**: SW55; **lane 3**: SW70; **lane 4**: DAI-enriched fraction from DEAE-cellulose chromatography; **lane 5**: DAI-depleted fraction from DEAE-cellulose chromatography. **Panel C** shows complex formation with:- **Lane 1**: ribosomal salt wash from HeLa cells; **lane 2**: ribosomal salt wash from HeLa cells treated with human α-interferons (1000 U/ml for 24h). **Panel D** shows:- **Lane 1**: complex formation with reticulocyte SW40 fraction; **lane 2**: as lane 1 but complexes treated with proteinase K as described in Materials and methods. The arrow-heads indicate the wells of the gels. To save space only the upper parts of the gels are shown in panels B–D.

Competition for complex formation

Competition for complex formation by a variety of unlabelled RNA species and other polyanions was used to examine the specificity of EBER-1 binding and to assess the requirements of the binding protein for EBER-1 sequence or secondary structure. Fig. 3 shows competition data using both the SW40 and DAI-enriched protein fractions. As expected, unlabelled EBER-1 effectively competed out labelled EBER-1 from the complex (Fig. 3A). Calf liver tRNA, heparin and *E. coli* 5S rRNA were unable to compete with labelled EBER-1 for binding (Fig. 3B and 3D), suggesting that complex formation was not due to non-specific RNA-protein interaction. Other RNAs transcribed *in vitro* from bacteriophage RNA polymerase promoters were also unable to compete, even at a 300-fold molar excess. This reinforces the conclusion that the binding was not due to contaminating dsRNA as this would be expected to exist in all preparations of bacteriophage RNA polymerase transcripts. A 142 nucleotide uncapped transcript corresponding to the 5′ end of rabbit β-globin mRNA, that is essentially single-stranded (30), could not compete for binding (Fig.3B), further suggesting that binding is sequence and/or structure-specific.

In order to test the requirement for secondary structure EBER-1 transcribed in the presence of ITP rather than GTP (i-EBER-1) was added to a competition assay. This RNA has a disrupted secondary structure due to the presence of inosine residues (36)

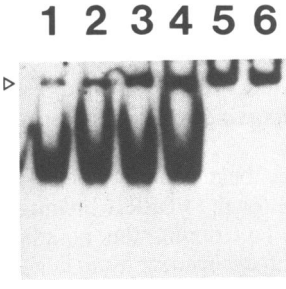

Figure 2. Effect on complex formation of preincubation of EBER-1 with ribonucleases. ^{32}P-EBER-1 was untreated (**lanes 1 and 2**) or preincubated with RNase III (14 U) (**lanes 3 and 4**) or RNases T_1 and A (10 U and 10 μg respectively) (**lanes 5 and 6**) for 15 min at 37°C in binding buffer. The samples were then incubated with reticulocyte SW40 salt wash fraction (**lanes 1, 3 and 5**) or the DAI-enriched fraction from DEAE-cellulose chromatography (**lanes 2, 4 and 6**) and analysed as described in Fig.1.

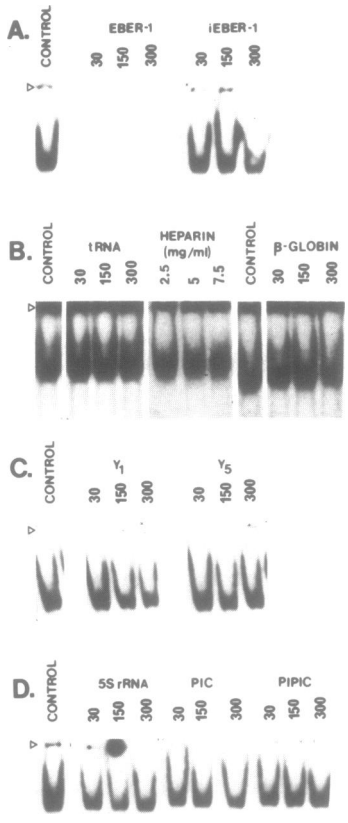

Figure 3. Competition for EBER-1-protein complex formation. ^{32}P-EBER-1 was incubated with either the DAI-enriched fraction from DEAE-cellulose chromatography (**panels A, C and D**) or the SW40 salt wash fraction (**panel B**) in the absence or presence of various unlabelled competing RNA species. **Panel A** shows competition by EBER-1 itself in comparison with inosine-substituted EBER-1 (iEBER-1). **Panel B** shows the results of competition assays using calf liver tRNA, heparin and a 142 nucleotide fragment from the 5′ end of rabbit β globin mRNA transcribed by SP6 RNA polymerase. **Panel C** shows the results of competition assays using the small cytoplasmic Y_1 and Y_5 RNAs transcribed by T7 RNA polymerase *in vitro*. **Panel D** shows the results of competition assays using *E.coli* 5S rRNA or poly(I).poly(C) added simultaneously with the EBER-1 (PIC) or preincubated with the protein fraction for 15 min at 30°C prior to addition of EBER-1 (PIPIC). The figures above each lane indicate the molar excess of competitor RNA over EBER-1, except in the case of heparin where the actual concentration (in mg/ml) is shown. The arrow-heads indicate the wells of the gels.

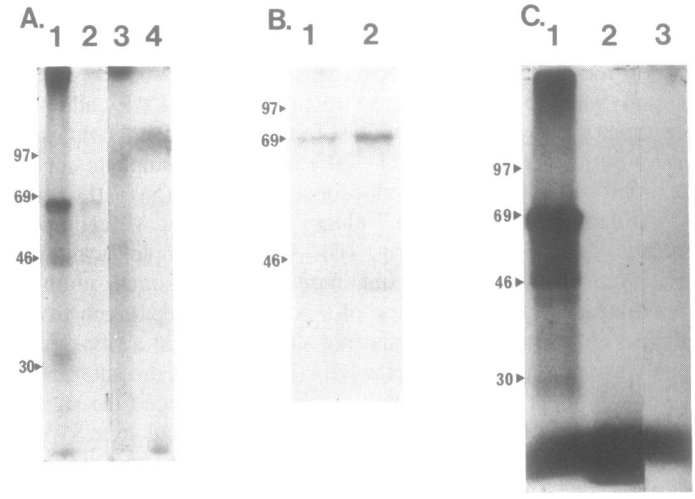

Figure 4. Formation of covalent EBER-1-protein complexes by UV crosslinking. ^{32}P-EBER-1 was incubated with reticulocyte protein fractions and covalently crosslinked by UV irradiation as described in Materials and methods. After digestion with RNases T_1 and A the samples were subjected to SDS gel electrophoresis and autoradiography. **Panel A** shows complexes formed with:- **Lane 1**: SW40 salt wash fraction; **lane 2**: SW55 fraction; **lane 3**: SW70 fraction; **lane 4**: unfractionated reticulocyte lysate. **Panel B** shows a separate experiment using:- **Lane 1**: SW40 fraction; **lane 2**: DAI-enriched fraction from DEAE-cellulose chromatography. **Panel C** shows complexes formed with:- **Lane 1**: SW40 fraction; **lane 2**: as lane 1 but treated with proteinase K after incubation; **lane 3**: as lane 1 but EBER-1 preincubated with RNases T_1 and A (10 U and 10 μg respectively) prior to addition of the SW40 fraction. The numbers to the left of each panel indicate the positions of molecular weight markers (sizes in kDa).

and cannot prevent the dsRNA-mediated inhibition of protein synthesis in an *in vitro* translation assay where, under the same conditions, EBER-1 prevents this inhibition (28). I-EBER-1 could only compete slightly for complex formation when added at a 300-fold molar excess over labelled EBER-1 (Fig. 3A), emphasising the importance of EBER-1 secondary structure for protein binding.

The La protein has been shown to bind EBER-1 (7) and was a possible candidate for the EBER-1 binding protein detected in these experiments. To examine this possibility we carried out a binding competition experiment using labelled EBER-1 and the two small La-binding RNA species, Y_1 and Y_5, transcribed by T7 RNA polymerase *in vitro* (plasmids were a gift from Dr W. van Venrooij). These RNAs are cellular cytoplasmic species of 112 and 84 nucleotides respectively. They were identified as binding to Ro proteins and both also carry the characteristic 3′ U terminus recognised by the La protein (37,38). Neither of these RNAs had the ability to compete with EBER-1 for complex formation (Fig. 3C). This absence of competition eliminates the La protein as a candidate for the EBER-1 binding protein detected in these assays. The Y RNAs have stem-loop structures similar to that of EBER-1 (29). Nevertheless their lack of competition indicates that the binding of EBER-1 must be very specific and the presence of stable stem-loops alone is not sufficient for complex formation.

The presence of the protein that could bind EBER-1 in various reticulocyte salt wash fractions, and our previous *in vitro* translation data (28), suggested that the protein might be DAI. This protein kinase is activated by low concentrations (10^{-9} to 10^{-8} g/ml) of dsRNA (39,40). We therefore examined whether a synthetic dsRNA, poly (I).poly (C), would be able to compete out EBER-1 binding. However, a preparation of poly(I).poly (C) that was active in inhibiting reticulocyte lysate protein synthesis did not compete, even when pre-incubated with the protein source prior to the addition of labelled EBER-1 (Fig. 3D).

UV-crosslinking of labelled EBER-1 to protein

Whilst the gel retardation experiments show that complex formation is very specific to EBER-1, they cannot identify the nature of the protein component of the complex. In order to determine whether the EBER-1 binding protein might be DAI, UV-crosslinking experiments using labelled EBER-1 and the protein sources described above were carried out. In the SW40 and DAI-enriched fractions labelled EBER-1 could be crosslinked to two proteins with sizes of approximately 64−68kDa and 46−53kDa, with the former giving the more intense signal (Fig.4A and B). Estimates of the sizes of the proteins vary somewhat, depending on the gel system and molecular weight markers used. In some experiments a faint band was also observed at about 31kDa. The 64−68kDa protein was also detected at much lower levels in the SW55 fraction but no

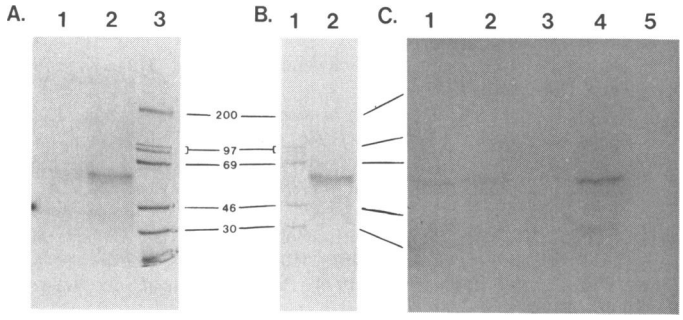

Figure 5. Immunoprecipitation of UV crosslinked complexes. ^{32}P-EBER-1 was incubated with the DAI-enriched fraction from DEAE-cellulose chromatography and UV crosslinked as described in Materials and methods. Immunoprecipitations were carried out using an anti-DAI guinea-pig serum or a control guinea-pig serum and protein A-agarose, as described in Materials and methods, and the samples were subjected to SDS minigel electrophoresis and autoradiography. **Panel A** shows the results obtained with:- **Lane 1**: control serum; **lane 2**: anti-DAI serum. **Lane 3** contains the molecular weight markers indicated (sizes in kDa). For comparison, **panel B** shows an SDS gel analysis of non-immunoprecipitated, crosslinked EBER-1-protein complexes (**lane 2**) run on the same minigel apparatus with the same set of molecular weight markers (**lane 1**). **Panel C** identifies the position and relative amounts of DAI as determined by immunoblotting with an anti-DAI monoclonal antibody followed by incubation with a biotinylated anti-mouse immunoglobulin and a streptavidin-alkaline phosphatase conjugate:- **Lane 1**: SW40 fraction; **lane 2**: SW55 fraction; **lane 3**: SW70 fraction; **lane 4**: DAI-enriched fraction from DEAE-cellulose chromatography; **lane 5**: DAI-depleted fraction from DEAE-cellulose chromatography.

significant binding was detected in the SW70 fraction or in whole reticulocyte lysate (Fig.4A). No complexes were observed if the EBER-1 was pre-incubated with RNases T_1 and A, or if proteinase K was added to the incubations after binding and crosslinking had occurred (Fig.4C). Competition for complex formation, carried out prior to crosslinking, gave essentially the same results as in the gel retardation experiments. Unlabelled EBER-1 could compete with labelled EBER-1 but heparin, the β globin mRNA 5′ sequence and poly(I).poly(C) could not (data not shown).

Identification of the proteins crosslinked to labelled EBER-1

The size and distribution of the specific EBER-1 binding protein(s) identified in the gel retardation and UV-crosslinking assays is consistent with the possibility that EBER-1 binds to DAI. To determine further whether the binding protein(s) correspond to DAI, labelled EBER-1 was crosslinked to protein as before and immunoprecipitated with a guinea-pig antiserum against DAI. Fig 5A shows that the antiserum immunoprecipitated a labelled EBER-1-protein complex of approximately 65kDa, together with a minor species of lower molecular weight. A control guinea-pig serum, used at the same concentration as the anti-DAI serum, produced only a faint signal which was probably due to non-specific binding to the protein A-agarose employed in the immunoprecipitations. The mobilities of the immunoprecipitated complexes on the minigel system used in this experiment were identical to the mobilities of the UV-crosslinked complexes described above (Fig.5B). Bands in similar positions on the gels were also identified by immunoblotting with a monoclonal antibody against DAI (Fig.5C). The relative content of DAI in the protein fractions correlates well with the ability of these fractions to form complexes with EBER-1 (Figs.1 and 4). These results therefore strongly suggest that the EBER-1 binding protein(s) is closely related or identical to DAI.

DISCUSSION

The data accumulated from these different experimental approaches demonstrate that EBER-1 binds to DAI *in vitro*. The EBER-1-protein complex was formed in all the fractions where DAI was expected to be present and not in the fractions where DAI is known to be absent; it was also more prominent in HeLa cell extracts after treatment of the cells with interferon (which induces DAI—ref. 41). In the case of unfractionated reticulocyte lysate only a faint signal was detected. A possible explanation for this is that the EBER-1 binding protein is not sufficiently concentrated to form a detectable complex in whole lysate. The fact that a complex with identical mobility is formed in fractions from rabbit reticulocytes, mouse erythroleukaemia cells and HeLa cells indicates that the protein component of the complex is not species or cell type-specific.

The binding of EBER-1 to DAI appears to be dependent on the secondary structure of the RNA, suggesting that DAI specifically recognizes short double-stranded regions within EBER-1. The minimal structural requirements for functional binding of adenovirus VA_I RNA to DAI have recently been defined (42,43), but it is not yet known whether a similar structure within EBER-1 is sufficient for interaction with the protein. The absence of competition by poly (I).poly (C) suggests that there may be more than one RNA binding site on DAI, allowing EBER-1 to bind equally well in the presence and absence of this dsRNA. A higher affinity of DAI for EBER-1 or VA_I RNA than for dsRNA cannot explain the lack of competition since the small RNAs are required in a large molar excess over dsRNA to prevent the activation of DAI *in vitro* (24,28). It has been reported that poly (I).poly (C) also cannot compete with labelled VA_I RNA for binding to purified DAI in an *in vitro* filter binding assay, and evidence for two RNA binding sites has been presented (24). Nevertheless, since another study identified only one RNA binding site on DAI (25) this question needs further investigation.

The detection of two complexes containing bound EBER-1, both of which are recognised by anti-DAI antibodies, suggests that the smaller one may be structurally related to the larger species of 64−68kDa. DAI was originally thought to contain two subunits of 68 and 48kDa (44) although cDNA sequence data indicate that it consists of a single polypeptide with a molecular weight of 62kDa (41). This protein migrates anomalously in some gel systems, giving it an apparent size of 68kDa. The 48kDa protein may arise either from partial proteolytic cleavage during extraction and purification (33) or from the use of a different translational start site on the mRNA (41).

The capacity of the different protein fractions tested for formation of crosslinked complexes, and the specificity of binding as indicated by the competition data, largely agree with the data obtained from the gel retardation experiments. The major difference between the two sets of data is the presence of only one identifiable complex in the gel retardation experiments. This probably corresponds to the larger form of DAI. However, if autoradiographs from the retardation experiments are grossly overexposed, a second complex with a higher mobility can be detected (data not shown) and this may correspond to the 48kDa protein.

These results and our previous observations (28) suggest that EBER-1 can function in a manner analogous to that of VA_I RNA *in vitro*, to prevent the activation of DAI by dsRNA. Nevertheless, since the life-cycles of EBV and adenoviruses are

very different, it is likely that the physiological roles of EBER-1 and VA_I RNA are not identical during virus infection. We have recently discussed possible functions for EBER-1 during EBV-induced immortalisation of B cells (45). Efforts must now be directed towards intact cell studies in order to define the role of EBER-1 in EBV infection *in vivo*.

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