JOURNAL OF VIROLOGY, Jan. 2003, p. 228–236 Vol. 77, No. 1
0022-538X/03/$08.00+0 DOI: 10.1128/JVI.77.1.228–236.2003

Identification of a Lytic-Cycle Epstein-Barr Virus Gene Product That Can Regulate PKR Activation

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Received 29 May 2002/Accepted 20 September 2002

The Epstein-Barr virus (EBV) SM protein is a posttranscriptional regulator of viral gene expression. Like many transactivators encoded by herpesviruses, SM transports predominantly unspliced viral mRNA cargo from the nucleus to the cytosol, where it is subsequently translated. This activity likely involves a region of the protein that has homology to the herpes simplex virus type 1 (HSV-1) ICP27 gene product, the first member of this class of regulators to be discovered. However, SM also contains a repetitive segment rich in arginine and proline residues that is dispensable for its effects on RNA transport and splicing. This portion of SM, comprised of RXP triplet repeats, shows homology to the carboxyl-terminal domain of Us11, a double-stranded RNA (dsRNA) binding protein encoded by HSV-1 that inhibits activation of the cellular PKR kinase. To evaluate the intrinsic ability of SM to regulate PKR, we expressed and purified several SM protein derivatives and examined their activity in a variety of biochemical assays. The full-length SM protein bound dsRNA, associated physically with PKR, and prevented PKR activation. Removal of the 37-residue RXP domain significantly compromised all of these activities. Furthermore, the SM RXP domain was itself sufficient to inhibit PKR activation and interact with the kinase. Relative to its Us11 counterpart, the SM RXP segment bound dsRNA with reduced affinity and responded differently to single-stranded competitor polynucleotides. Thus, SM represents the first EBV gene product expressed during the lytic cycle that can prevent PKR activation. In addition, the RXP repeat segment appears to be a conserved herpesvirus motif capable of associating with dsRNA and modulating activation of the PKR kinase, a molecule important for the control of translation and the cellular antiviral response.

Early in the course of a lytic infection, Epstein-Barr virus (EBV) expresses a polypeptide 479 amino acids in length known as SM, Mta, or EB2 that regulates viral gene expression posttranscriptionally (9, 13, 29). The SM polypeptide, encoded by a spliced mRNA that joins the BSLF2 and BMLF1 open reading frames, can transactivate reporter gene expression in a promoter-independent, gene-specific fashion in transient-transfection assays (4, 9, 25, 29, 41, 42). In addition, SM has been reported to bind RNA, shuttle into and out of the nucleus, inhibit expression of intron-containing genes, and activate expression of genes that lack introns (2, 3, 8, 13, 18, 41, 42, 45). Many EBV genes important for viral replication in productively infected cells do not contain introns, and the cytoplasmic accumulation of their mRNAs is regulated by SM, as are cytoplasmic levels of mRNAs transcribed from reporter genes that do not contain introns (13, 26, 45). Other studies, however, provide evidence that SM inhibits splicing of precursors containing weak 5′ splice site consensus sequences and mediates their accumulation in the cytosol (3, 18, 45). Finally, SM associates with the cellular SC35 splicing factor (8, 26, 45).

Many of these activities are thought to involve a carboxyl-terminal region homologous to the herpes simplex virus type 1 (HSV-1) ICP27 polypeptide. A number of human herpesviruses encode posttranscriptional transactivators similar to ICP27 that are thought to bind RNA, mediate the transport of nascent viral transcripts from the nucleus to the cytosol, and perhaps regulate splicing as well (1, 2, 14, 15, 21, 27, 43, 47, 49). The EBV SM protein is unique among these in that it also possesses a module rich in arginines and prolines that is not required for its effects on splicing and RNA transport (3, 41). This element is similar to one found in the HSV Us11 polypeptide, an RNA binding protein that prevents activation of the cellular PKR kinase (38, 39, 44).

As a pivotal component of the host's innate antiviral response, double-stranded RNA (dsRNA) or highly structured RNA abundant in virus-infected cells activates PKR (reviewed in references 24, 28, and 37). Subsequently, the activated kinase phosphorylates the alpha subunit of eIF2, resulting in inactivation of the cellular translation initiation factor and inhibition of protein synthesis. Many viruses encode functions that regulate eIF2α phosphorylation because failure to counteract this response could result in the premature cessation of viral protein synthesis and prevent the production of proteins required to assemble infectious viral progeny (reviewed in reference 37).

Among human herpesviruses, human cytomegalovirus, Kaposi's sarcoma-associated herpesvirus, EBV, and HSV-1 all contain genes that are important for preventing the accumulation of phosphorylated eIF2α (5, 10–12, 22, 33, 46). Cells latently infected with EBV, for example, produce large amounts of small noncoding EBER RNAs that bind to PKR and prevent activation of the enzyme (11, 12, 46). HSV-1 employs a different strategy and targets multiple steps in this signaling pathway. While the γ34.5 gene product complexes with the cellular protein phosphatase 1α (PP1α) to dephos-

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phorylate eIF2α and maintain the active form of the translation initiation factor, the Us11 protein acts to prevent PKR activation (7, 22, 33, 38). The carboxyl-terminal 68 amino acids of Us11 associate with polyribosomes in the cytosol, accumulate in the nucleolus, prevent PKR activation, interact with PKR, and bind RNA (6, 38–40, 44). The Us11 RNA binding domain is not homologous to any known RNA binding motifs, and recent studies demonstrate that it binds to dsRNA (26a). Furthermore, it is composed of the sequence Arg-X-Pro repeated approximately 21 times, depending on the strain. A similar RXP repeat is found in the EBV SM polypeptide.

While the small, noncoding EBER RNAs produced by EBV in latently infected cells can prevent PKR activation, they are not synthesized during lytic replication, when the potential to form viral dsRNA is greatest (19, 20). Since the SM protein is expressed early in the EBV lytic cycle and contains an RXP motif, we investigated if SM could function in a manner analogous to the Us11 protein. This work demonstrates that SM binds PKR and inhibits activation of the PKR kinase; moreover, the isolated RXP domain is required for this effect. This is the first identification of a lytic cycle EBV gene product that can regulate PKR activation.

MATERIALS AND METHODS

Expression vectors and plasmids. Plasmid p128.5, containing the *Cla*-C fragment from EBV strain B95-8 (GenBank accession no. VO1555), was a generous gift from Bill Sugden (McArdle Laboratory for Cancer Research, Madison, Wis.). A cDNA encoding the full-length SM protein was reconstructed as described previously (13). The full-length SM open reading frame (codons 1 to 479; nucleotides 84288 to 84229 adjoined to nucleotides 84122 to 82743) was fused to the carboxyl terminus of glutathione *S*-transferase (GST) in plasmid pGEX-4T1 to create pGEX-SM. Deleting the RXP segment specified by codons 148 to 184 created the mutant derivative pGEX-SM-ΔRXP. SM codons 148 to 184 were fused to the carboxyl terminus of GST in pGEX-4T1 to generate pGEX-SM-RXP.

To engineer an SM protein that contains six His residues at its amino terminus (His tagged), the full-length SM open reading frame was inserted into a pET-15b variant lacking the thrombin cleavage site. A deletion removing codons 148 to 184, which specify the RXP repetitive region, was incorporated into pET-15b SM to generate pET-15b-SMΔRXP. To create recombinant baculoviruses expressing either His-tagged SM or His-tagged SMΔRXP, pET-15b-SM and pET-15b-SMΔRXP were digested with *Xba*I and *Hin*dIII. The resulting fragment containing the SM open reading frame from each construct was isolated and transferred into pFAST-Bac1. Recombinant baculoviruses were constructed with the Bac-to-Bac expression system (Life Technologies) according to the manufacturer's instructions. GST fusion proteins were purified by affinity chromatography on glutathione-agarose (Pharmacia), and His-tagged polypeptides were isolated with Ni-nitrilotriacetic acid-agarose (Qiagen). Eluted protein fractions were pooled, dialyzed into 20 mM HEPES (pH 7.4)–100 mM KCl–0.5 mM dithiothreitol, quick-frozen in small aliquots, and stored at −80°C.

PKR binding assays. A total of 100 μl of a 10% glutathione–agarose slurry equilibrated in SMPB (20 mM HEPES-KOH [pH 7.4], 100 mM KCl, 5 mM magnesium acetate, 1.5 mM dithiothreitol) was mixed with various quantities of purified GST fusion proteins for 1 h. on ice, with periodic agitation. The beads were subsequently collected by centrifugation and suspended in 1 ml of SMPB plus 3% fraction V bovine serum albumin. After rocking for 1 h at room temperature, the beads were collected by centrifugation, suspended in 0.1 ml of SMPB plus 3% fraction V bovine serum albumin, 0.5% Triton X-100, and 1 mM $CaCl_2$ and treated for 20 min at room temperature with 8 U of micrococcal nuclease per ml or 8 U of micrococcal nuclease, 50 μg of RNase A, and 300 U of RNase T_1 per ml.

^{35}S-labeled PKR produced by translation in vitro according to the manufacturer's directions (wheat germ T7 TNT quick coupled transcription-translation system; Promega, Madison, Wis.) was likewise treated with nuclease at the conclusion of the synthesis reaction. Phenylmethylsulfonyl fluoride was then added to both the beads loaded with GST fusion protein and the nuclease-treated in vitro translation reaction to a final concentration of 100 μM. Then 1 μl of the in vitro-translated lysate was added to the nuclease-treated beads containing bound fusion protein, and the final concentration of phenylmethylsulfonyl fluoride was brought to 200 μM. Binding reaction mixtures were incubated at 30°C for 30 min and agitated manually every 5 min. The beads were subsequently collected by centrifugation and washed three times with 1 ml of SMPB plus 0.5% Triton X-100. Each wash was rocked for 3 min at room temperature prior to centrifugation. The final pellets were resuspended in 20 μl of 1× Laemmli sample buffer, and the bound proteins were resolved by electrophoresis in a sodium dodecyl sulfate (SDS)–10% polyacrylamide gel. Gels were impregnated with a fluorophore prior to drying (Research Products International) and exposed to Kodak XAR film.

Preparation of labeled substrates and dsRNA binding assays. All polynucleotides were from Pharmacia. Poly(IC) was 5′ end labeled with T4 polynucleotide kinase (New England Biolabs) according to the manufacturer's instructions. Unincorporated nucleotides were removed on a Biogel P30 spin column (Bio-Rad). Increasing amounts of purified GST fusion proteins were assembled on ice with labeled poly(IC) (approximately 28,000 cpm/reaction) in 1× binding buffer (20 mM HEPES [pH 7.4], 50 mM NaCl, 1 mM EDTA, 5% glycerol) plus 1 mg of yeast RNA (Boehringer) per ml and 50 μg of nuclease-free bovine serum albumin per ml. After 15 min at 30°C, the reaction mixtures were applied to a manifold (Bio-Rad) containing a nitrocellulose sheet (Protran, 0.45-μm pore size; Schleicher and Schuell) that had been equilibrated in 1× binding buffer. Each sample was subsequently washed three times with 0.2 ml of 1× binding buffer. Filters were air dried and exposed to Kodak XAR film, and the radioactivity present in the excised spots was quantified by Cerenkov counting. For competition studies performed in the presence of excess unlabeled polynucleotides, purified proteins were the final component added to the assembled reaction mixes.

To prepare labeled dsRNA, plasmid pBlueScript SKII(+) was linearized with *Bam*HI, and an 81-nucleotide RNA was synthesized in vitro with T7 RNA polymerase in the presence of [α-^{32}P]CTP. This was annealed to a 245-nucleotide RNA synthesized by T3 RNA polymerase from *Pvu*II-digested pBlueScript SKII(+). Single-stranded regions were trimmed by treating with RNases A and T_1, and an 81-bp RNA duplex was isolated as described previously (30). Each filter binding reaction mixture contained 5,000 cpm of labeled substrate.

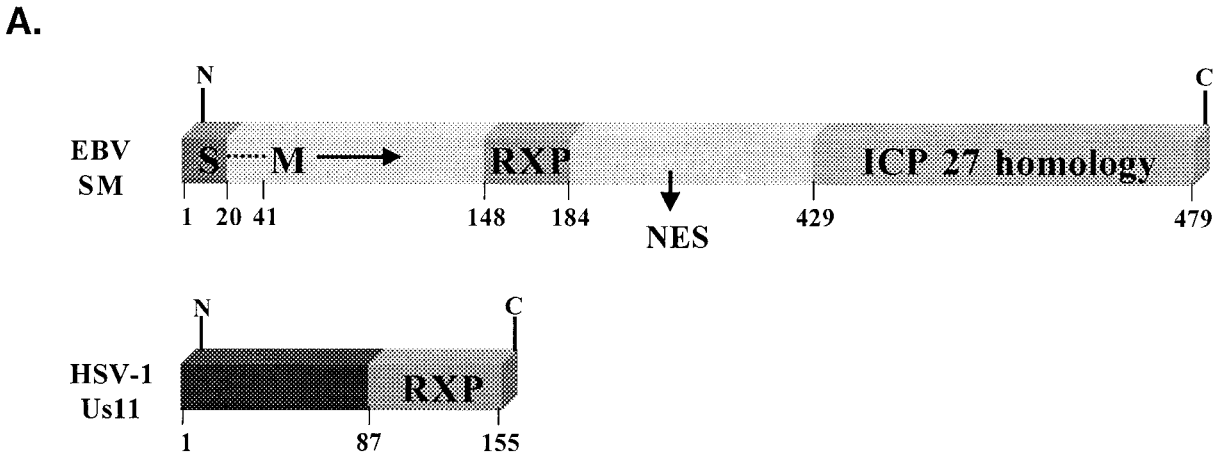
Preparation of S10 extracts and PKR kinase assay. Preparation of S10 extracts and the PKR kinase assay were performed as described by Poppers et al. (38).

Immunochemical reagents and procedures. The anti-His tag monoclonal antibody was from Novagen. PKR immunoprecipitations were performed with affinity-purified rabbit polyclonal antiserum (sc707; Santa Cruz Biotechnology). For Western analysis, proteins were transferred from SDS-polyacrylamide gels onto a polyvinylidene difluoride membrane (Immobilon-P; Millipore). Immobilized antigen-antibody complexes were detected by chemiluminecence (ECL kit; Amersham-Pharmacia).

RESULTS

Repetitive RXP motif present in alpha- and gammaherpesvirus RNA binding proteins. SM contains multiple functional regions that could represent independently folded domains. The carboxyl-terminal segment exhibits homology to several herpesvirus posttranscriptional regulators, including the ICP27 gene product, an immediate-early protein encoded by the alphaherpesvirus HSV-1. Together with the nuclear export signal, this element is likely to be important for transporting mRNA from the nucleus to the cytosol (2, 3, 8, 18, 45). It may also be required for the observed effects on RNA processing (3, 18, 45). In addition, SM possesses an RNA binding domain rich in arginine and proline that binds to a variety of unrelated RNAs.

The EBV BMRF1 and BMLF1 RNAs associate with SM, while unrelated cellular RNAs do not (45). However, SM also can interact with mRNA transcribed from several transiently transfected reporter genes (3, 41, 42). RNA segments derived from the 3′ untranslated region of the HSV-1 UL38 gene, the EBV VCA capsid antigen gene, and a variety of reporter genes bind to SM as well (42). The SM RNA binding domain and the



B.

HSV-1 (Patton)	PRVPREPRVPRPPREPREPRVPRLPRDPRVPRDPRDPRQPRSPREPRSPREPR------TPRTPREPRTARGSV
HSV-1 (Strain 17)	PRVPREPRVPRPPREPREPRVPRAPRDPRVPRDPRDPRQPRSPREPRSPREPRSPREPRTPRTPREPRTARGSV
HSV-2 (HG52)	PRVPREPRVP---REPREPRVPRSPREPRVPRIPRDPRPPR-P--PRVPREPR---------PPREPRATRGLA
Simian B virus	PRPPREPRTPRRPRPPRQPREPRQPRVPREPREPRQPREPRQPRRPRVPREPRVPRTPRHHVQPPAPHDPRG--
EBV SM	ARAPRAPRAP---------RVPRAPRSPRAPRSNRATRGP

FIG. 1. EBV SM protein and Us11 polypeptide encoded by alphaherpesviruses both contain a repetitive RXP segment. (A) Domain structure of the SM and Us11 proteins. The 479-amino-acid SM polypeptide is encoded by a spliced mRNA that joins a segment of the BSLF2 open reading frame (S; amino acids 1 to 20) to the BMLF1 open reading frame (M; amino acids 41 to 479). A dotted line between the S segment and the M open reading frame represents the 20 residues encoded by sequences immediately upstream of the BMLF1 ATG codon. The RXP segment, along with the nuclear export sequence (NES) and ICP27 homology domain, is shown. The HSV-1 Us11 protein (Patton strain) is 155 amino acids long and contains a 68-amino-acid RXP domain in its carboxyl terminus. (B) Sequences of the RXP segments from SM appear aligned with various alphaherpesvirus Us11 proteins (HSV-1 Patton strain, HSV-1 strain 17, HSV-2 strain HG52, and simian virus B).

RNA binding domain of the HSV-1 Us11 protein both contain multiple copies of the amino acid sequence RXP. The Us11 carboxyl-terminal region includes 21 copies of this triplet motif, while SM contains 8 repeat units (Fig. 1). In addition, the amino acid that occupies the central position in the RXP triplet varies in Us11 compared to SM. Whereas the X residue is frequently hydrophobic or acidic in Us11, it is often alanine in SM. Presently, Us11 and SM are the only proteins in the database that contain this unique repetitive RXP motif; moreover, the only sequence homology between Us11 and SM resides within their respective RXP modules.

RXP segment promotes binding of SM to dsRNA. Recently, we established that the Us11 RXP domain comprises a novel dsRNA recognition motif (26a). To determine if the EBV SM protein can also interact with dsRNA, we expressed and purified several SM protein derivatives and examined their ability to associate with dsRNA substrates in a filter binding assay. Upon incubation with labeled poly(IC), a synthetic dsRNA polymer, increasing quantities of a purified GST fusion protein containing the 37-amino-acid RXP segment from SM bound poly(IC) in a dose-dependent fashion (Fig. 2A). Addition of equivalent quantities of GST alone did not result in retention of radioactivity on the nitrocellulose filter (Fig. 2A).

Although the isolated SM RXP motif bound dsRNA, it did not bind as well as the 68-amino-acid RXP domain from the HSV-1 Us11 protein. Discernible levels of protein-RNA complexes were detected with as little as 3 ng of GSTΔ1-87, while at least 100 ng was required to detect binding with the RXP segment from SM (Fig. 2A and data not shown). At higher protein concentrations, the association of the EBV GST-RXP protein with poly(IC) was enhanced, as it bound only 3- to 3.6-fold less dsRNA than the Us11-derived fusion protein (Fig. 2A and data not shown). While the SM RXP segment fused to GST is shorter than the related Us11 domain, it is composed of only eight RXP iterations, whereas the corresponding domain in Us11 contains 21 copies. Furthermore, there is little conservation among the X residues in the two motifs.

A peculiar feature of the binding of the Us11- and SM RXP-derived segments to poly(IC) was their behavior in the presence of excess unlabeled polyribonucleotides. Inclusion of unlabeled poly(A) or poly(U) in the reactions has little effect on the association of GST SM-RXP with poly(IC), while poly(IC) and poly(C) competed effectively for binding to the labeled substrate (Fig. 2B and C). This could reflect an intrinsic preference of the SM RXP domain to associate with RNA molecules that contain one or more tracts of C-rich sequence in addition to its dsRNA binding activity. Furthermore, low levels of poly(C) reproducibly stimulated binding to labeled poly(IC) by a factor of up to 3 (Fig. 2C). In contrast, binding of GSTΔ1-87 to poly(IC) was unaffected by excess poly(C),

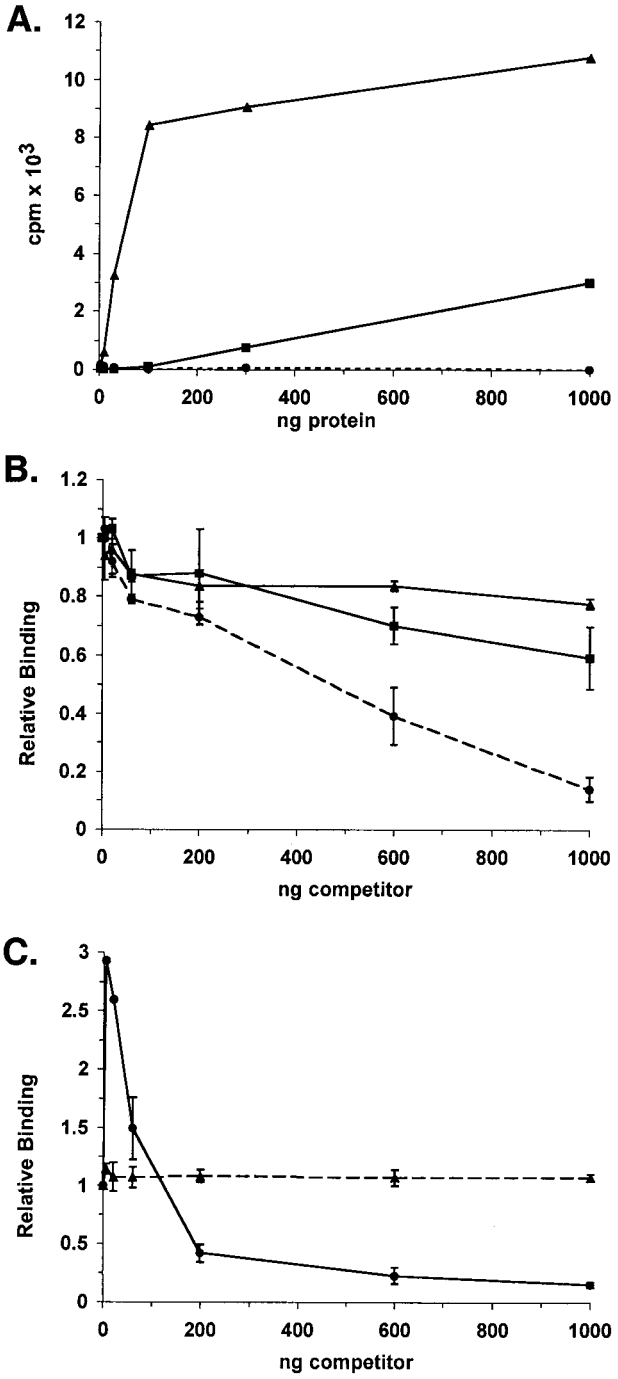


FIG. 2. Binding of herpesvirus RXP domains to poly(IC). (A) Increasing quantities of purified GST or purified proteins containing either the HSV-1 Us11 RXP domain (GSTΔ1-87) or the EBV SM RXP segment (GST-SM-RXP) fused to GST were incubated with labeled poly(IC), and the amount of radioactivity retained after filtering the reaction mixtures through nitrocellulose was quantified. GSTΔ1-87, ▲; GST-SM-RXP, ■; GST, ●, broken line. (B) GST-SM-RXP (300 ng) was incubated with labeled poly(IC) (approximately 4 ng) in the presence of increasing amounts of excess unlabeled poly(IC) (●, broken line), poly(A) (▲), or poly(U) (■). The amount of radioactivity retained on a nitrocellulose filter in the absence of unlabeled competitor was normalized to 1.0. (C) GSTΔ1-87 (10 ng) (▲, broken line) or GST-SM-RXP (300 ng) (●) was incubated with labeled poly(IC) in the presence of increasing amounts of unlabeled poly(C). Binding was quantified and normalized as described for panel B.

poly(U), or poly(A) and was only reduced in the presence of unlabeled poly(IC) (Fig. 2C) (26a). Thus, although both RXP segments are sufficient to interact with poly(IC), they do so with different affinities and exhibit distinct responses upon challenge with competitor polynucleotides.

Poly(IC) is a synthetic RNA polymer that is extremely heterogeneous in size. To evaluate binding to dsRNAs of uniform size that exhibit a more natural sequence complexity, we prepared an 81-bp dsRNA molecule from in vitro-synthesized transcripts derived from the pBlueScript cloning vector. In addition, to assess the dsRNA binding properties of the full-length protein, we constructed recombinant baculoviruses that expressed His-tagged wild-type SM (His-SM) or a mutant SM that lacked the 37-amino-acid RXP segment (His-ΔRXP). While the overall yield of the baculovirus-expressed protein was lower than what we were able to obtain by producing these polypeptides in bacteria, the quality of intact protein was superior (Fig. 3A and unpublished observations). Figure 3B demonstrates that increasing quantities of full-length SM did indeed bind dsRNA; moreover, this interaction was reduced by four- to fivefold upon removal of the RXP segment at the highest amount of protein tested (Fig. 3B). The SM RXP motif is therefore important for binding to dsRNA, and its deletion diminishes this interaction.

The 37-amino-acid RXP domain from SM is sufficient to interact with PKR. Recent studies have demonstrated that a segment of Us11 containing a portion of the RXP repetitive region is important for binding to PKR (6). To determine if the EBV SM polypeptide interacts with PKR, we incubated purified GST-SM or GST-Us11 derivatives that were immobilized on glutathione-agarose beads with in vitro-translated, [^{35}S]methionine-labeled PKR. Prior to mixing, both the protein-bound beads and the in vitro-translated PKR were treated with micrococcal nuclease or a combination of micrococcal nuclease, RNase A, and RNase T_1 to degrade double-stranded and single-stranded nucleic acids in the reaction mixture. This procedure was effective at reducing the amount of acid-insoluble, radiolabeled RNA [either poly(IC) or a synthetic 335-nucleotide RNA] by over 99% (not shown).

After the collected beads had been washed, the bound proteins were fractionated by electrophoresis in SDS-polyacrylamide gels, and the amount of labeled PKR was visualized by autoradiography. Beads containing the 30-amino-acid SM RXP motif fused to GST efficiently retained PKR, while beads loaded with GST alone did not interact with PKR (Fig. 4A). This demonstrates that the 30-amino-acid RXP segment derived from SM is sufficient to bind to PKR. The 68-amino-acid RXP motif from Us11 was also sufficient to bind PKR under identical conditions. Beads containing GST fused to the amino-terminal 87 amino acids of Us11, however, did not associate with PKR (Fig. 4A).

The interaction between the RXP domains and PKR appears to be specific, as other in vitro-translated proteins, one of which also binds nucleic acid, were not retained on the beads (Fig. 4B). Finally, the interaction between herpesvirus RXP-containing proteins and the cellular PKR kinase was resistant to treatment with micrococcal nuclease, RNase A, and RNase T_1 (Fig. 4 and 5 and data not shown).

Association of SM with PKR requires RXP domain. To determine if the RXP motif in the context of the intact SM

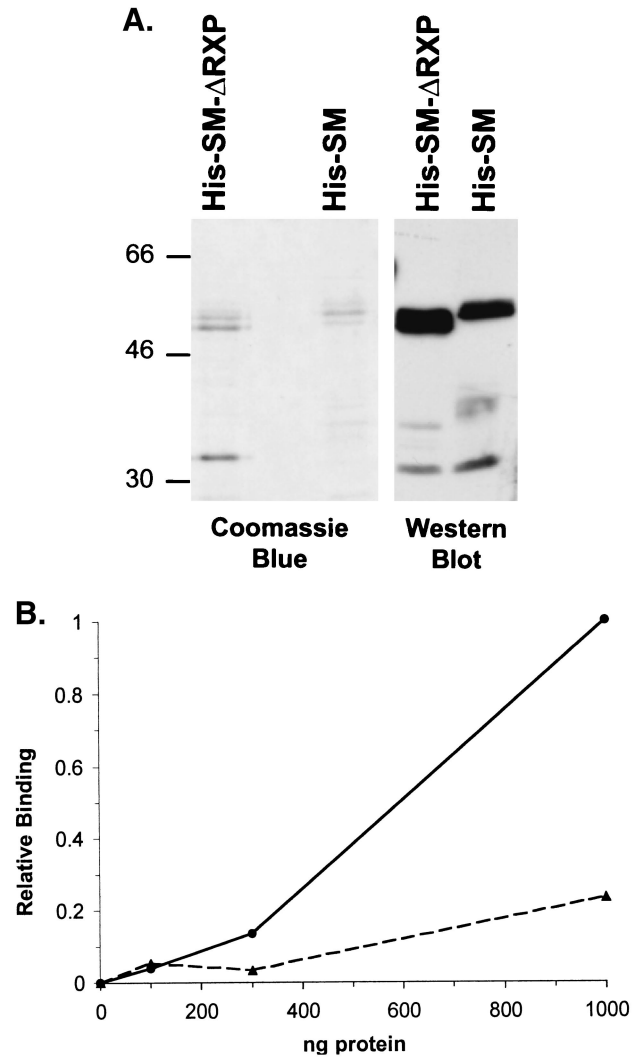


FIG. 3. RXP segment embedded in full-length SM protein is important for dsRNA binding. (A) Full-length His-tagged SM (His-SM) and a mutant derivative that lacks the RXP domain (His-SM-ΔRXP) were isolated from cells infected with recombinant baculoviruses. Purified proteins were subjected to electrophoresis in SDS-polyacrylamide gels and either stained with Coomassie brilliant blue R-250 or transferred to a membrane which was subsequently probed with an anti-His monoclonal antibody. Positions of molecular size markers are shown (in kilodaltons). (B) A labeled 81-bp RNA duplex was prepared and incubated with increasing amounts of either His-SM (●) or His-S-ΔRXP (▲, broken line). Binding to dsRNA was measured by quantifying the radioactivity retained on the nitrocellulose filter.

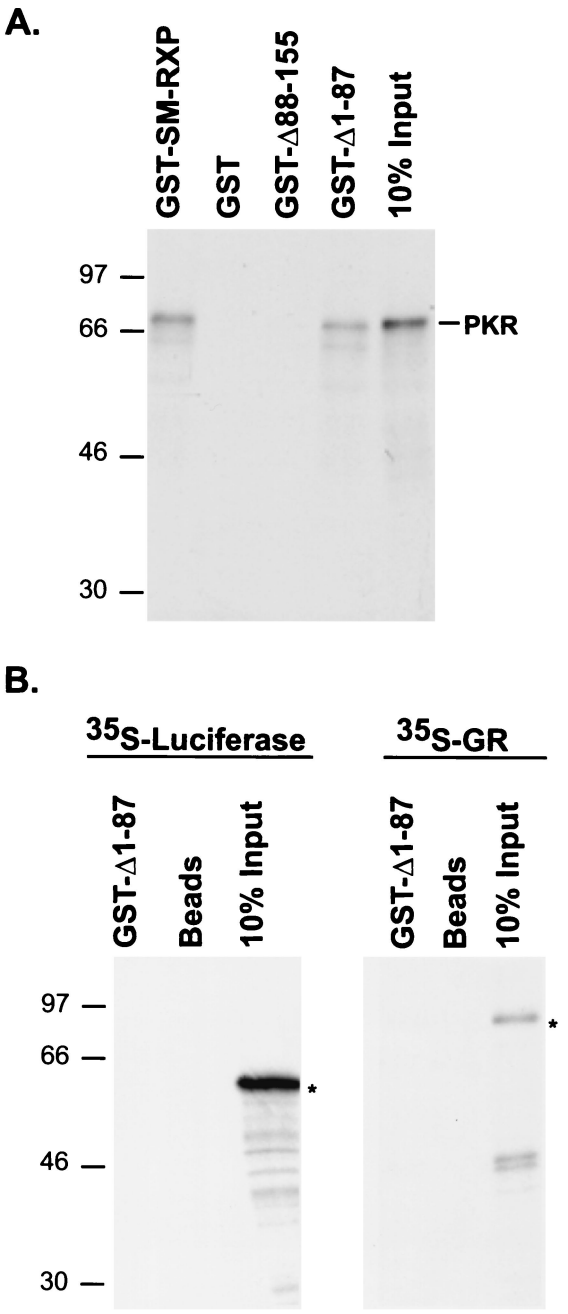


FIG. 4. SM RXP domain is sufficient to interact with PKR. Purified GST fusion proteins bound to glutathione-agarose were incubated with in vitro-translated ^{35}S-labeled PKR. Prior to mixing, both the lysate and the immobilized GST fusion proteins were treated separately with micrococcal nuclease. After the beads were collected and washed, the bound proteins were fractionated in SDS-polyacrylamide gels that were subsequently impregnated with a fluorophore and exposed to film. Positions of molecular size markers are indicated to the left (in kilodaltons). (A) GST-SM-RXP contains the 37-amino-acid segment from SM fused to GST; GSTΔ1-87 contains the carboxyl-terminal 68 amino acids from Us11 fused to GST; and GSTΔ88-155 contains the amino-terminal 87 amino acids of Us11 fused to GST. (B) The RXP domain does not interact nonspecifically with in vitro-translated luciferase or the glucocorticoid receptor. Full-length translation products are indicated with an asterisk to the right of each image.

polypeptide was capable of forming a complex with PKR, we fused a full-length reconstructed SM cDNA to the coding sequences for GST and purified the expressed protein from bacteria. In addition, we constructed a deletion mutant that did not contain the 37-amino-acid RXP segment (GST-SMΔRXP). Figure 5 demonstrates that PKR was detected in a complex with full-length GST-SM. Removal of the RXP domain, however, severely reduced this association (Fig. 5). It is important to note that due to the large differences in molecular weights, 300 ng of GST-RXP and 1 μg of GST-SM each contained approximately 10 pmol of the RXP domain and bound

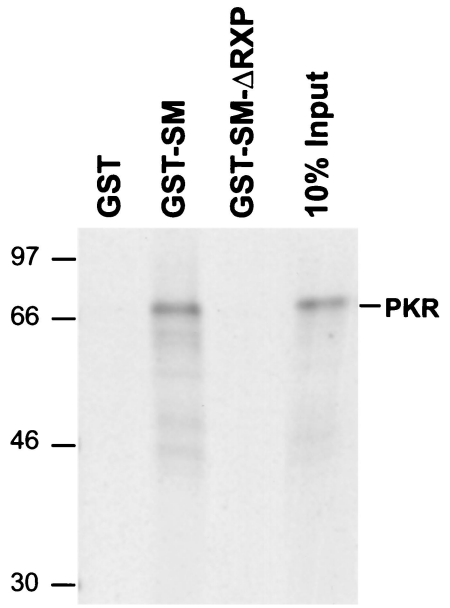


FIG. 5. Interaction of full-length SM with PKR requires the RXP repetitive segment. Purified GST fusion proteins were bound to glutathione-agarose and treated as described in the legend to Fig. 4. GST-SM contains amino acids 1 to 479 of SM fused to GST; GST-SM-ΔRXP contains an internal deletion of 37 amino acids that removes the RXP segment.

PKR equally well (data not shown). Thus, PKR can associate with the full-length SM polypeptide, and this interaction is largely dependent on the RXP module.

SM RXP domain can modulate PKR activation. Previously, we established that the 68-amino-acid RXP module from Us11 was sufficient to prevent PKR activation (38). To evaluate the ability of the SM RXP module to inhibit PKR activation, purified GST or GST-RXP fusion proteins were added to cell extracts prepared from alpha interferon-stimulated 293 cells. PKR can be activated in these extracts by the addition of dsRNA, and the extent of activation can be monitored by immunoprecipitating PKR from reaction mixes that contain [γ-^{32}P]ATP. Following fractionation of proteins in the immune complex on SDS-polyacrylamide gels, activated PKR can be visualized by autoradiography and quantified by phosphorimager analysis.

Inclusion of increasing quantities of GST-RXP in the reaction mixture resulted in a dose-dependent reduction in the amount of activated PKR, while the addition of GST had no effect (Fig. 6A). At the highest dose of GST-RXP evaluated, this inhibition represented a 3.6-fold decrease in the amount of phosphorylated PKR compared to that in control reaction mixtures that were programmed with GST alone. Thus, the 37-amino-acid RXP motif from the EBV SM protein is sufficient to inhibit PKR activation.

Interestingly, one-third of the mass of the 68-amino-acid Us11 RXP domain is required to inhibit PKR activation to the same extent as a fixed quantity of the SM RXP motif (30) (data not shown). As Us11 contains approximately three times more RXP repeats than SM, it appears that similar levels of inhibition are achieved by comparable concentrations of RXP units.

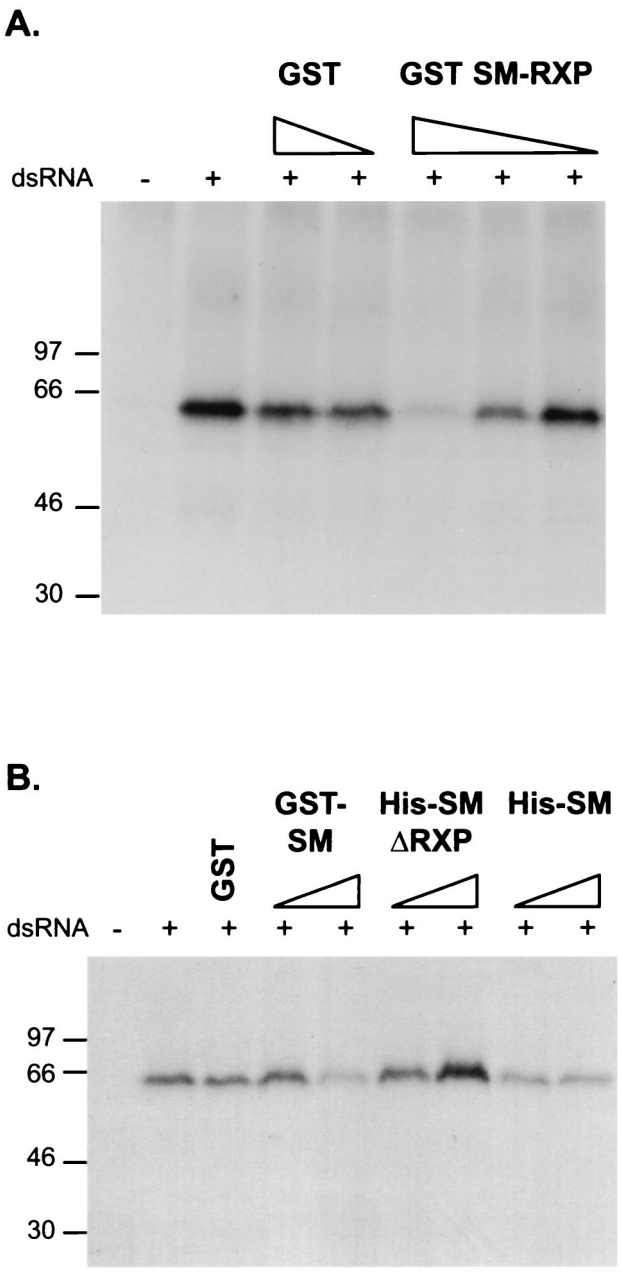


FIG. 6. SM can regulate PKR activation. (A) The SM RXP segment is sufficient to inhibit PKR activation. Increasing amounts of either purified GST-RXP (9, 6, 32, or 85 pmol) or GST (37 or 98 pmol) were incubated in S10 extracts containing [γ-^{32}P]ATP in the presence or absence of dsRNA. Following incubation at 30°C for 30 min, PKR was immunoprecipitated, and the immune complexes were fractionated in an SDS-polyacrylamide gel. The fixed, dried gel was subsequently exposed to film. Positions of molecular size markers are indicated to the left (in kilodaltons). (B) Removal of the RXP segment from SM reduces its ability to inhibit PKR activation. Same as in panel A except that the reaction mixtures contained purified GST-SM (3.75 or 12.5 pmol), His-SM (5.7 or 19 pmol), His-SM-ΔRXP (5.7 or 19 pmol), or GST (37 pmol). Positions of molecular size markers are indicated to the left (in kilodaltons).

Finally, the full-length SM protein also prevents PKR activation, as purified, His-tagged SM reduced the amount of phosphorylated PKR 1.7- to 3.5-fold compared to a His-tagged variant that lacks the RXP domain (Fig. 6B). Similarly, addition of purified GST-SM decreased levels of phosphorylated PKR by approximately twofold compared to addition of GST alone (Fig. 6B). Thus, the RXP domain in SM is required to prevent activation of PKR.

DISCUSSION

Although SM regulates gene expression posttranscriptionally during the EBV lytic cycle, it executes at least two independent functions involving RNA metabolism. Previous studies have shown that SM shuttles between the nuclear and cytoplasmic compartments, transporting predominantly unspliced viral RNA cargo (2, 3, 8, 13, 18, 26, 41, 42, 45). This activity is exemplified by a class of herpesvirus-encoded molecules that show homology to the HSV-1 ICP27 transactivator (1, 2, 14, 15, 21, 27, 43, 47, 49). We now report that SM can prevent activation of the cellular PKR kinase, an eIF2α kinase capable of inhibiting protein synthesis in response to dsRNA and viral infection. The repetitive RXP sequence contained in SM associates with PKR, binds dsRNA, and is required to inhibit PKR activation. Us11, an RNA binding protein encoded by HSV-1, also contains a repetitive RXP domain that directs a similar array of activities (6, 26a, 38). Thus, the RXP motif defines a new domain in herpesvirus proteins that is designed to interact with PKR and dsRNA. Consequently, polypeptides that contain RXP elements are able to prevent PKR activation, blocking a strategic component important for the host's antiviral response.

Presently, the RXP module is found only in the Us11 protein specified by several alphaherpesviruses and the SM protein encoded by EBV, a gammaherpesvirus. This domain could have been acquired independently by both virus types or could have been captured initially by the alphaherpesviruses prior to the divergence of the gammaherpesvirus family. It is important to recognize that the RXP-containing proteins are otherwise unrelated, as SM, in fact, shows structural and functional homology to an additional alphaherpesvirus protein, the herpes simplex virus ICP27 polypeptide (2, 3, 8, 13, 18, 42, 45). Similar transactivator proteins encoded by other herpesviruses do not contain an RXP repetitive element, and Us11-homologous proteins are found only in HSV-1, HSV-2, and the simian B virus (16, 31, 35). Importantly, however, several human herpesviruses are known to encode activities that can regulate PKR activation regardless of whether they produce an RXP-containing protein (5, 10).

While the RXP segments from SM and Us11 inhibit PKR activation to similar extents, the Us11 domain has a much greater affinity for poly(IC). The 21 RXP repeats in Us11 compared to the 8 copies in SM likely contribute to this difference, as do variations in the identity of the amino acid in the central position of the triplet repeat. It does, however, call into question the role of dsRNA binding by the RXP element in preventing PKR activation. In this regard, the 68-amino-acid Us11 RXP domain prevents PKR activation by PACT, a cellular protein that activates the kinase in the absence of dsRNA (37a). Although RXP-containing herpesvirus proteins may inhibit PKR activation in the absence of dsRNA, that does not necessarily mean that dsRNA binding is not important. The RXP domain could effectively sequester dsRNA molecules and prevent them from activating the kinase. The effectiveness of different RXP-containing proteins in operating via the latter mechanism may reflect their affinity for dsRNA.

Whereas both ICP27 and SM are posttranscriptional regulators of gene expression, delivering RNA molecules to the cytosol, where they are subsequently translated, the 37-amino-acid RXP segment embedded in SM is not required for this activity (3, 42). This strongly implies that there is another mechanism through which SM associates with RNA, either through a second RNA recognition domain or via interaction with another protein that can bind RNA. Interestingly, earlier studies demonstrated RNA binding only for SM derivatives which contained the RXP element; however, although the RXP segment was required for RNA binding in a Northwestern assay, the full-length protein did not bind to a chloramphenicol acetyltransferase reporter RNA probe (42). Only truncated derivatives were capable of binding RNA, fueling the speculation that the ability of the RXP domain to recognize RNA could be regulated.

In our dsRNA filter binding assay, removal of the RXP motif impaired dsRNA binding, although some detectable activity still remained in the ΔRXP mutant. Indeed, posttranslational modifications or interactions with other ligands could change the conformation of SM, altering the accessibility of the RXP segment and the efficiency with which it recognizes RNA. Intriguingly, poly(C) stimulated binding to poly(IC), suggesting that single-stranded RNA might modulate the dsRNA binding activity of the SM RXP domain. Consolidating these two functions in a single polypeptide or domain could provide a mechanism to sequester dsRNA regions in viral mRNA transcripts, effectively shielding them from PKR.

The RXP domain in SM is required for complex formation with PKR. While this interaction resists extensive treatment with micrococcal nuclease, RNase A, and RNase T_1, we cannot absolutely rule out the possibility that a small piece of nuclease-resistant, structured RNA somehow facilitates this protein-protein interaction. Recently, it has been reported that Us11 interacts with PKR and that this association is completely disrupted by treatment with RNases A and T_1 (6). It is possible that the different techniques used in these two investigations could be important in understanding this discrepancy. In their study, Cassady and Gross demonstrated that Us11 can be detected in anti-PKR immune complexes isolated from HSV-1-infected cells and that this interaction is disrupted by prior treatment with RNases A and T_1 (6). However, those authors used affinity-purified antipeptide antiserum to immunoprecipitate PKR and were therefore only isolating complexes in which this epitope was exposed. If, upon associating with PKR in the absence of RNA, this epitope becomes masked via a change in conformation or by steric hindrance, the complex would not be recognized by the antiserum. Perhaps two forms of Us11-PKR complexes can form in cells, one mediated by nucleic acids and one independent of nucleic acid. Such a scenario is not without precedent, as PKR interacts with the cellular protein NF90 through RNA-dependent and -independent associations (36).

EBV encodes at least two functions that inhibit activation of the cellular PKR kinase. Earlier studies demonstrated that the

small, nonpolyadenylated EBER RNAs could prevent PKR activation in a manner akin to the VA RNAs produced by adenovirus (11, 12, 46). Recently, however, it has become clear that the EBERs are synthesized in latently infected cells (19, 20). The EBERs can inhibit PKR activation in response to a repetitive region in the 5′ untranslated region of viral latent transcripts; in addition, they may contribute to the observed resistance of EBV-associated tumor cells to apoptosis induced by alpha interferon (17, 34). Paradoxically, the EBERs are not required for viral lytic replication or transformation of cultured cells (48).

In a manner analogous to that of other herpesviruses, EBV probably produces dsRNA during its productive growth cycle as well and would therefore need to express lytic cycle viral functions to counteract dsRNA-dependent signal pathways that have a negative effect on viral replication (22, 23, 32, 33). Our identification of a conserved motif within the SM transactivator represents the first description of a lytic cycle EBV gene product that can prevent PKR activation. Furthermore, it indicates that EBV, like HSV-1, possesses multiple independent functions designed to hinder PKR-mediated signaling and thereby obstruct the cellular antiviral response. The generation of recombinant EBV genomes harboring viable mutant SM alleles which do not affect other, potentially essential functions of the polypeptide will enable further evaluation of these activities in cells productively infected with EBV.

ACKNOWLEDGMENTS

We are grateful to Bill Sugden for providing EBV genomic DNA, Aaron Shatkin for supplying reovirus RNA, Ganes Sen for the gift of the PKR expression plasmid, and Michael Garabedian for the glucocorticoid receptor expression plasmid.

This work was supported by a grant from the NIH to I.M. An NIH training grant partially supported J.P.

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