The latency protein LANA2 from Kaposi’s sarcoma-associated herpesvirus inhibits apoptosis induced by dsRNA-activated protein kinase but not RNase L activation

M. Esteban,1 M. A. García,1 E. Domingo-Gil,1 J. Arroyo,2 C. Nombela2 and C. Rivas2

1Departamento de Biología Celular y Molecular, Centro Nacional de Biotecnología, Consejo Superior de Investigaciones Científicas (CSIC), Campus Universidad Autónoma de Madrid, 28049 Madrid, Spain
2Departamento de Microbiología II, Facultad de Farmacia, Universidad Complutense de Madrid, Plaza Ramón y Cajal sn, 28040 Madrid, Spain

INTRODUCTION

Virus infection induces expression of a group of proteins, the interferons (IFNs), which play major roles as a first-line host defence response against pathogens. IFNs are multifunctional cytokines with important roles in the induction of antiviral, cell growth, differentiation and immunomodulatory functions (Harada et al., 1998; Stark et al., 1998). There are two types of IFN, type I (IFN-α/β) and type II (IFN-γ). Type I IFNs are produced by virus-infected cells and constitute the primary response against virus infection, whereas type II IFN is a Th1 cytokine produced by activated T cells and natural killer cells and is involved in immune regulation (Vilcek & Sen, 1996; Stark et al., 1998). Induction of IFN is highly regulated through IFN regulatory factors (IRFs), a family of transcription factors with a broad range of activities (Nguyen et al., 1997; Mamane et al., 1999; Taniguchi et al., 2001). The different biological actions of IFN are mediated by the products of specifically induced cellular genes in target cells. Two of these IFN-regulated cellular products are the dsRNA-activated protein kinase (PKR) and the 2-5A system. PKR controls cell growth (Chong et al., 1992), cell differentiation (Petrushyn et al., 1984), virus clearance (Lee & Esteban, 1993; Lee et al., 1996) and induction of apoptosis (Lee & Esteban, 1994). PKR is a serine/threonine kinase activated by dsRNA binding and exerts its effect on subsequent autophosphorylation. Some targets of the activated PKR are the phosphorylation of the eukaryotic translation initiation factor 2 (eIF-2α) and NFκB activation, events that mediate the induction of apoptosis by PKR. Activation of the caspase 9 pathway together with the fas-associated death domain (FADD)/caspase 8 pathway are also events downstream of PKR-induced apoptosis, although this activation is not required for the induction of apoptosis (Gil et al., 2002). The 2-5A pathway is constituted by the IFN-induced proteins 2-5A synthetase and 2-5A-dependent RNase L, together with 2-5A phosphodiesterase. dsRNA activates the 2-5A synthetases that, in the presence of ATP, synthesize a complex mixture (referred to as 2-5AS) of 2’5’-triphosphorylated oligoadenylic acid, ppp(A2’p5)nA, which binds to and activates RNase L for cleavage of viral and cellular RNAs, resulting in general inhibition of protein synthesis (Samuel, 1991). These events correlate with antiviral and pro-apoptotic activities.

Since the IFN-induced cellular antiviral response is the primary defence mechanism against virus infections, many viruses have evolved mechanisms to counteract the effects of IFN (Vilcek & Sen, 1996; Goodbourn et al., 2000). In this sense, viruses have developed multiple strategies to avoid the deleterious effects of the PKR and 2-5A system at different levels (Gale & Katze, 1998), such as inhibition of PKR.
activation (Katze et al., 1987), sequestration of dsRNA (Lloyd & Shatkin, 1993; Lu et al., 1995), inhibition of PKR dimerization (Gale et al., 1997, 1998), synthesis of PKR pseudosubstrates (Dever et al., 1998; Taylor et al., 1999), activation of antagonist phosphatases (He et al., 1997) or PKR degradation (Black et al., 1989).

Kaposi’s sarcoma-associated herpesvirus (KSHV), or human herpesvirus-8 (HHV-8), is recognized as the essential infectious agent linked to the development of Kaposi’s sarcoma, primary effusion lymphoma and some forms of Castleman’s disease, an atypical B cell lymphoproliferative disorder (Chang et al., 1994; Soulier et al., 1995). The genome of KSHV contains cellular homologues that permit the manipulation of the local environment for efficient virus replication and evasion of the immune response and may also contribute to host proliferation and cell transformation. These cellular homologues include a cluster of open reading frames (ORFs) encoding four proteins with homology to the cellular transcription factors of the IRF family. vIRF1 inhibits virus-mediated transcriptional activation of the IFNa gene promoter and IFN-stimulated activation of ISG promoters (Gao et al., 1997; Li et al., 1998; Zimring et al., 1998; Buryseck et al., 1999). Furthermore, expression of vIRF1 also activates KSHV genes such as vIL6 and inhibits radiation and adriamycin-induced apoptosis in KSHV-infected cells (Sarid et al., 1999). Another KSHV IRF-like protein, vIRF2, inhibits the antiviral effect of IFN and rescues translation of vesicular stomatitis virus mRNA from the IFN-induced translation block. vIRF2 interacts physically with PKR and inhibits its autophosphorylation, thus blocking phosphorylation of eIF-2α (Burysek & Pitha, 2001). Finally, the ORFK10.5 encodes the LANA2 protein (previously called vIRF6) with homology to cellular IRF-4 and KSHV vIRF2 (Rivas et al., 2001). LANA2 functions as a dominant-negative mutant of both IRF-3 and IRF-7 and inhibits virus-mediated transcriptional activity of the IFNa promoter (Lubyova & Pitha, 2000).

KSHV has thus developed strategies to counteract IFN through the synthesis of several viral proteins with homology to the IRF-like proteins. However, only one KSHV protein (vIRF2) has been identified so far that specifically counteracts PKR action, the most common target of virus action to block IFN signalling pathways. In this investigation, we have examined the ability of KSHV LANA2 to modulate the dsRNA-activated pathways, PKR and the 5′-5A system. We found that LANA2 was specifically able to inhibit apoptosis and the blockade in translation mediated by PKR. These effects may be mediated by a decrease in eIF-2α phosphorylation but not by direct interaction between LANA2 and PKR.

METHODS

Antibodies and reagents. Polyclonal rabbit antibody specific for PKR has been previously described (Gil et al., 1999). Mouse monoclonal serum specific for LANA2 was kindly provided by Y. Chang and P. Moore (Hillman Cancer Center, University of Pittsburgh School of Medicine, PA, USA). Antiserum specific for the phosphorylated form of eIF-2α was purchased from Research Genetics. Anti-caspase 9 was from Oncogene. Caspase 9 inhibitors were from Calbiochem. Monoclonal β-actin antibody was obtained from Sigma.

Cells and viruses. African green monkey kidney cell line BSC-40 was grown in Dulbecco’s modified medium (DMEM) supplemented with 10% newborn calf serum. Mouse fibroblasts derived from homozygous PKR knockout mice were obtained from C. Weissmann (Imperial College, London, UK) and grown using the same media. Cells were cultivated at 37°C with 5% CO2. The recombinant vaccinia virus (VV) VV-Rl, VV-ΔN and VV-2-5A5 have been described previously (Díaz-Guerra et al., 1997a). The recombinant virus VV-PKR expresses PKR under ladd repressor/operator controlling elements (Lee & Esteban, 1993). A VV recombinant expressing the p36/LACK protein of Leishmania infantum VVP36 has been described previously (Gonzalo et al., 2001). LANA2 was extracted from the EGP-LANA2 plasmid and subcloned into the SmaI site of the haemagglutinin insertional VV vector PHLZ (Vazquez & Esteban, 1999). The EGP-LANA2 plasmid was generated by cloning the DNA encoding LANA2 excised from the pDNA-LANA2 plasmid (kindly provided by Y. Chang and P. Moore) and inserted into the EGP vector (Clontech) as a KpnI–ApaI fragment. VV-LANA2 was generated by homologous recombination of PHLZ-LANA2 with wild-type VV Western Reserve strain (WR) in BSC-40 cells and selected by blue plaque formation in response to addition of X-Gal. Virus was subjected to five rounds of plaque purification to generate homogeneous population of recombinants, grown and titrated in BSC-40 cells.

Analysis of low molecular mass DNA. Low molecular mass DNA was isolated from cultured cells as described (Hirt, 1967), analysed by agarose gel electrophoresis and stained with ethidium bromide.

Measurement of the extent of apoptosis. The Cell Death Detection ELISA kit from Boehringer Mannheim was used according to the manufacturer’s instructions. This assay, based on the quantitative sandwich-enzyme-immunoassay principle using mouse monoclonal antibodies directed against DNA and histones, respectively, estimates the amount of cytoplasmic histone-associated DNA.

Total RNA isolation. Total cellular RNA from mock-infected or infected cells was isolated using the Ultraspec-II Resin Purification System (Biotecx). For analysis, denatured RNA was fractionated on 1% formaldehyde/agarose gels and stained using ethidium bromide (Díaz-Guerra et al., 1997b).

Analysis of protein synthesis. For immunoblot analysis, protein samples were fractionated by SDS-PAGE, transferred to nitrocellulose paper and analysed by immunoperoxidase staining after reactivity with different sera.

Metabolic labelling of proteins. BSC-40 cells cultured in 24-well plates were infected with the viruses indicated and rinsed three times with methionine/cysteine (Met/Cys)-free DMEM 30 min prior to labelling. After incubation for an additional 30 min at 37°C with Met/Cys-free DMEM, medium was removed and 25 μCi [35S]Met/Cys Pro-mix (Amersham) ml−1 in Met/Cys-free DMEM was added for an additional 30 min. After three washes with PBS, cells were harvested in lysis buffer followed by SDS-PAGE and autoradiography. Experiments were repeated at least twice.

Immunoprecipitation. For in vivo co-immunoprecipitations, BSC-40 cells were grown in 10 cm plates and infected for 24 h with VV-LANA2 and/or VV-PKR. Cells were scraped and the clarified supernatant was mixed with 150 μl of protein A–Sepharose, previously incubated with specific antibodies directed against LANA2 or PKR, and further incubated overnight at 4°C. Immunocomplexes were resolved by SDS-PAGE followed by immunoblot analysis with anti-PKR and anti-LANA2 antibodies.
Measurement of β-Gal activity. Confluent BSC-40 cells seeded in 24-well plates were infected with the indicated viruses and 5 mM IPTG was added to induce PKR expression. Cells were collected 24 h post-infection (p.i.), resuspended in 100 μl 0.25 M Tris, pH 7.8, and lysed by three freeze–thaw cycles. Lysis extracts were diluted to 1 ml with water, centrifuged and 10 μl of supernatant were used for β-Gal determination. Cell lystate supernatants were mixed with 150 μl of CPRG solution (1 mM MgCl₂, 45 mM β-mercaptoethanol, 0.1 M sodium phosphate, pH 7.5, 5 mM CPRG) in a 96-well plate, incubated at 37°C for 1 h and the absorbance at 540 nm determined.

Caspase 3 activity assay. Cells (2 × 10⁶) were collected in lysis buffer (150 mM KCl, 10% glycerol, 1 mM DTT, 5 mM magnesium acetate, 0-5% Nonidet P-40) and clarified by centrifugation. Equal amounts of supernatant and 2 × reaction buffer (100 mM HEPES, pH 7.5, 20% glycerol, 5 mM DTT, 0.5 mM EDTA) were mixed and assayed for caspase 3 activity using 200 μM DEVD-pNA (Calbiochem) as substrate. Free pNA produced by caspase activity was determined by measuring absorbance at 405 nm.

NFκB activation assay. Mouse fibroblasts derived from homozygous PKR knockout mice were mock-infected or double-infected with 2 p.f.u. per cell WR or VV-LANA2 and up to 6 p.f.u. per cell of WR or VV-LANA2. At 24 h p.i., whole-cell extracts were prepared and processed following the manufacturer’s instructions for the NFκB p65 Transcription Factor Assay kit (Active Motif Europe).

RESULTS

Expression of LANA2 from recombinant VV
In order to generate a recombinant VV expressing LANA2, the LANA2-encoding region was subcloned into the insertional VV vector PHIZ and shuttled into the VV genome. Recombinant VV-LANA2 was then generated to allow the constitutive expression of LANA2 from the HA locus under the control of a VV early/late synthetic promoter (Fig. 1a). The recombinant VV-LANA2 expressed a protein of the correct size as determined by Western blot analysis in comparison with a positive control (Fig. 1b).

LANA2 does not inhibit the 2-5A synthetase/RNase L system
Two of the functional activities of the 2-5A synthetase/RNase L system are the induction of RNA degradation and the triggering of apoptosis (Castelli et al., 1997; Dı´az-Guerra et al., 1997b; Zhou et al., 1997). In order to determine the ability of the LANA2 protein to inhibit this IFN-induced pathway, we carried out co-infections with VV vectors that express RNase L, 2-5A synthetase and LANA2. In the case of RNase L co-infections, we used a VV vector that expresses T7 RNA polymerase, since RNase L was under control of the T7 promoter. In the case of VV-2-5AS, the 2-5AS synthetase was constitutively expressed under the control of an early/late virus promoter. All infections were made up to 6 p.f.u. per cell by adding wild-type WR virus. BSC-40 cells were infected with 2 p.f.u. per cell of each VV plus a VV vector expressing RNase L (VV-RL) in combination with wild-type VV (WR) or the recombinant VV expressing LANA2 (VV-LANA2). In addition, infections were carried out as described but in the presence of 2 p.f.u. per cell of the recombinant VV-2-5AS, which increases the effect of RNase L on apoptosis. The integrity of total RNA was examined by size fractionation on gels at various times after infection and apoptosis was determined by DNA ladder formation. Co-expression of LANA2 did not inhibit apoptosis mediated by the 2-5A synthetase/RNase L system (Fig. 2a) and did not affect the cleavage of RNA (Fig. 2b) at any time p.i.

LANA2 inhibits PKR-induced apoptosis
In order to determine the ability of the LANA2 protein to inhibit PKR-induced apoptosis, BSC-40 cells were infected with a recombinant VV expressing PKR in combination with either WR or VV-LANA2. Co-expression of LANA2 together with PKR partially inhibited the capacity of PKR to induce apoptosis, as measured by DNA ladder formation (Fig. 3a). The extent of apoptosis was quantified by analysis of oligonucleosomal cytoplasmic complexes. Inhibition of PKR-induced apoptosis by LANA2 was more pronounced with time of infection (Fig. 3b). These results suggested that LANA2 is able to interfere with apoptotic effects triggered by PKR activation.

Since PKR induces NFκB activation (Kumar et al., 1994; Gil et al., 2000), the effect of LANA2 over PKR-induced NFκB activation was analysed. LANA2 was unable to inhibit NFκB activation in response to PKR (data not shown).

LANA2 affects translational control induced by PKR
One of the major biological functions of PKR is to control translation through phosphorylation of eIF-2α (reviewed by Gale & Katze, 1998). Since LANA2 appears to affect
apoptosis induced by PKR, we next determined the effect of LANA2 on the inhibition of protein synthesis induced by PKR. BSC-40 cells were infected with VV-PKR in combination with WR or VV-LANA2 and de novo protein synthesis was determined by labeling with $[^{35}\text{S}]$methionine at 24 h p.i. As shown in Fig. 4(a), PKR severely inhibited protein synthesis in the presence of IPTG and this inhibition was partially prevented after co-infection with the recombinant expressing LANA2. To provide further evidence for the rescue of the translational control, we measured expression of $\beta$-Gal. This was achieved due to the presence of the lacZ gene in the viral genome of the recombinant viruses VV-PKR or VV-LANA2, which provides a visual marker for the isolation of the recombinant vectors. Thus, to quantify the effect of LANA2 on the extent of inhibition of viral protein synthesis by PKR, $\beta$-Gal activity was measured in extracts from cells infected with VV-PKR in combination with the recombinant virus VV-LANA2 or the recombinant virus VVp36, which also contains the lacZ gene in the viral genome and was used as a control. As shown in Fig. 4(b), inhibition of $\beta$-Gal activity by PKR expression was rescued by increasing amounts of LANA2. Thus, LANA2 was able to overcome the translational control induced by PKR over a single protein such as $\beta$-Gal.

Since the translational block induced by PKR is largely due to phosphorylation of elf-2x, we analysed the effect of LANA2 on PKR-mediated elf-2x phosphorylation. As shown in Fig. 4(c), the levels of elf-2x phosphorylated by
PKR decreased in the presence of increasing amounts of LANA2.

**LANA2 does not interact with PKR**

To study whether the effect of LANA2 on the eIF-2α phosphorylation induced by PKR was mediated by a direct interaction between LANA2 and PKR, *in vivo* binding assays were performed as described in Methods. We could not detect any direct interaction between the proteins, suggesting there is no physical interaction between LANA2 and PKR (data not shown).

**LANA2 inhibits caspase 3 but not caspase 9 activation**

PKR induces apoptosis by activation of the FADD/caspase 8 pathway. In addition, although dispensable, caspase 9 is also a mediator of PKR-induced cell death (Gil et al., 2002). Both apoptotic pathways converge in the final activation of terminator caspases, such as caspase 3. In view of the relevance of caspases in PKR-induced cell death, we decided to study whether LANA2 was able to block the activation of caspases 3 and 9 induced by PKR. Increasing amounts of LANA2 did not reduce the activation of caspase 9 triggered by PKR, as observed by the appearance of a smaller-sized protein (denoted with an arrow in Fig. 5a). However, increasing amounts of LANA2 decreased the levels of caspase 3 activation as shown in Fig. 5(b). These results suggest that only the FADD/caspase 8 pathway is affected by LANA2.

**DISCUSSION**

IFNs play a critical role in the innate immunity to virus infections. To overcome the inhibitory effect of IFNs, some viruses have developed a variety of strategies by which they can antagonize effects of the cytokines that are involved in virus clearance (Kunzi & Pitha, 1998; Ploegh, 1998). Several proteins have been involved in initiating cell death in response to virus infections and the 2-5A system and PKR are examples of those host defence proteins specifically activated by dsRNA, an intermediate in the virus replication-cycle (reviewed by Stark et al., 1998).

KSHV contains several homologues of cellular IRFs (Moore et al., 1996), having evolved a mechanism by which it directly subverts the functions of IRFs and down-regulates the induction of the IFN genes that are important components of the innate immunity (Lubyova & Pitha, 2000). However, only one of the KSHV IRF-like proteins, vIRF2, has been demonstrated to target the PKR protein specifically (Burysek & Pitha, 2001). The aim of this study was to investigate whether LANA2, another IRF-like protein from KSHV, with homology to vIRF2 (Rivas et al., 2001), had some effect on the two IFN-induced pathways, the 2-5A synthetase/RNase L system and PKR.

Activation of the IFN-induced enzyme RNase L causes RNA breakdown as well as apoptosis of animal cells, an effect that is enhanced by co-expression of 2-5A synthetase (Castelli et al., 1997; Diaz-Guerra et al., 1997b; Zhou et al., 1997). Analysis of RNA stability or apoptosis induced by activation
of the 2-5A system in the presence of LANA2 indicated that LANA2 did not interfere with this IFN-induced pathway. PKR conditions cellular apoptosis in response to activation by various stimuli such as dsRNA accumulated as a by-product of virus replication (Der et al., 1997), or when it is overexpressed (Lee & Esteban, 1994). Studies on the effect of LANA2 on PKR activity demonstrated that LANA2 inhibited apoptosis induced by PKR activation. Two of the cellular substrates of PKR are eIF-2α, which on phosphorylation abrogates translation initiation (Rowlands et al., 1988), and IκBα, the inhibitor of the transcription factor NFκB (reviewed by Baldwin, 1996). Activation of NFκB by PKR was not altered after LANA2 expression, suggesting that the activity of LANA2 did not affect this pathway. However, analysis of the levels of the phosphorylated form of eIF-2α after PKR activation in the presence of increasing amounts of LANA2 showed an inhibitory effect of LANA2 on eIF-2α phosphorylation. The decrease in the levels of phosphorylated eIF-2α may explain how LANA2 relieves the block of translation induced by PKR. However, we could not detect direct interaction between the proteins, suggesting that LANA2 activity is not executed through direct interaction with PKR. It is of interest to note that MC159L protein from molluscum contagiosum virus inhibits apoptosis induced by PKR in the absence of direct interaction but cannot block phosphorylation of eIF-2α (Gil et al., 2001), indicating different modes of action of these viral proteins that antagonize PKR activity.

Two apoptotic pathways in mammalian cells are employed to destroy virus-infected cells: the extrinsic death-receptor pathway and the intrinsic mitochondrial pathway. Both pathways converge to activate caspases, in particular the effector caspase 3 (Thornberry & Lazebnik, 1998). PKR induces apoptosis by activation of the FADD/caspase 8 pathway. In addition, although dispensable, caspase 9 is also a mediator of PKR-induced cell death (Gil et al., 2002). Our experiments measuring the effect of LANA2 on PKR-induced caspase activation revealed that LANA2 could inhibit PKR-induced activation of caspase 3 but not caspase 9, suggesting that only the FADD/caspase 8 pathway is affected by LANA2.

In conclusion, our findings show that KSHV encodes, in addition to the described vIRF2, another IRF-like protein, LANA2, that targets PKR. In contrast to vIRF2 (Burysek & Pitha, 2001), LANA2 is not a direct PKR inhibitor; however, it is able to target the eIF-2α phosphorylation pathway with the ability to revert the translational block imposed by PKR. Since both LANA2 and vIRF2 are KSHV latent proteins (Burysek & Pitha, 2001; Rivas et al., 2001), their cooperation to inhibit PKR activity could be a key mechanism in KSHV persistence.

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Inhibition of PKR by LANA2


