KSHV vFLIP binds to IKK-γ to activate IKK

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Summary
When expressed in heterologous cells, the viral FLIP protein (vFLIP) of Kaposi's-sarcoma-associated herpesvirus (KSHV) has been reported both to block Fas-mediated apoptosis and to activate the NF-κB activation pathway by interaction with IκB kinase (IKK). In a yeast-two-hybrid screen, we identified IKKγ as an interacting partner of vFLIP. We expressed fragments of IKKγ in mammalian cells and bacteria, and identified the central CCR3/4 (amino acids 150-272) as the vFLIP binding region. To investigate the proteins interacting with vFLIP in a KSHV-infected primary effusion lymphoma (PEL) cell line, we immunoprecipitated vFLIP and identified four associated proteins by mass spectrometry: IKK components IKKα, β and γ, and the chaperone, Hsp90. Using gel filtration chromatography, we demonstrated that a single population of vFLIP in the cytoplasm of PEL cells co-eluted and co-precipitated with an activated IKK complex. An inhibitor of Hsp90, geldanamycin, inhibited IKK's kinase activity induced by vFLIP and killed PEL cells, suggesting that vFLIP activation of IKK contributes to PEL cell survival.

Key words: KSHV, vFLIP, IKK, Hsp90

Introduction
Kaposi’s-sarcoma-associated herpesvirus (KSHV) encodes a viral FLIP protein (vFLIP) in open reading frame 71 (Orf71). vFLIP is expressed as one of a cluster of three latency associated genes that regulate proliferation and apoptosis (Dittmer et al., 1998; Fakhari et al., 2002; Jenner et al., 2001). The genes encoding LANA, vCyclin and vFLIP are transcribed together to produce LANA and LT1, respectively, with 50% sequence identity. IKK contains two catalytic components, IKKα and IKKβ (also called IKK1 and IKK2) (DiDonato et al., 1997; Mercurio et al., 1999) [also called NF-κB essential modulator (NEMO) (Yamaoka et al., 1998), IKK-associated protein 1 (IKKAP1) (Mercurio et al., 1999) and 14.7-interacting protein (FIP-3) (Li et al., 1999)]. IKKα and IKKβ are homologous proteins of 85 kDa and 87 kDa, respectively, with 50% sequence identity. IKKγ is necessary for activation of IKKα and IKKβ (Makris et al., 2000); heterodimers of IKKα and IKKβ are bound by four IKKγ molecules to form a large complex (Tegethoff et al., 2003). Recently, the chaperone protein Hsp90 and a co-chaperone (Cdc37) have been identified as additional components of the IKK complex (Chen et al., 2002).

KSHV infection is associated with three proliferative disorders in immune-compromised patients: Kaposi’s sarcoma (KS), primary effusion lymphoma (PEL) (a proliferation of immature B cells) and a variant of multicentric Castleman’s disease (MCD) (Boshoff et al., 2002; Cesarman et al., 1995; Moore et al., 1996; Soulier et al., 1995). In KSHV-infected PEL cells, the NF-κB pathway is constitutively active (Liu et al., 2002; Keller et al., 2000) and the cells undergo apoptosis...
when challenged with the inhibitor of cytokine-inducible IkBα phosphorylation, Bay 11-7082 (Keller et al., 2000). This suggested a role for constitutive NF-κB activation in the survival of these cells. Given the two contrasting roles previously assigned to vFLIP, we set out to investigate which proteins interact with vFLIP in KSHV-infected PEL cells.

Materials and Methods

Plasmids

Wild-type and mutant IkBα (S32A/S36A) plasmids were generous gifts from N. Perkins (Dundee, UK). Glutathione-S-transferase (GST)-IkBα fusion proteins containing the first N-terminal 54 amino acids of wild-type and mutant IkBα were constructed in pGEX-KT. HIV-1-based plasmids were kindly provided by D. Trono (Geneva, Switzerland) and are described elsewhere (Naldini et al., 1996; Zufferey et al., 1997). The vector, pHRCMV-eGFP contains a cytomagalovirus (CMV)-driven emerald green fluorescent protein (eGFP). To construct a vector expressing both vFLIP and eGFP, a sequence containing EMCV IRES and eGFP was amplified by PCR and introduced into the Xhol site of pHRCMV-LacZ to produce pHRCMV-LacZ-IRES-eGFP. The LacZ gene was then replaced with vFLIP. IKKβ truncation mutants were generated by PCR amplification of a human expressed sequence tag and subsequent cloning of the DNA fragments into the pcDNA4 mammalian expression vector (Invitrogen) downstream of an Xpress epitope tag or into pGEX-KT downstream of GST.

Cell lines and lentiviral transduction

The KSHV-infected PEL cell line, BC3, was grown in RPMI1640 with 10% foetal calf serum (FCS), penicillin and streptomycin at 37°C in 5% CO2. 293T cells were maintained in Dulbecco’s modified Eagle’s medium with 10% FCS, penicillin and streptomycin at 37°C in 10% CO2. Lentivirus encoding vFLIP and GFP or GFP alone was produced using a transient transfection of 293T cells as described previously (Neil et al., 2001; Zufferey et al., 1997). 293T cells were transduced with each virus and the efficiency of cell transduction measured by FACSscan analysis of eGFP positive cells. Cells were treated with 0.5 μM geldanamycin (GA) (Calbiochem) dissolved in DMSO or an equal volume of DMSO in serum-free medium for 16 hours.

Large-scale immunoprecipitation

Anti-vFLIP 6/14 rat monoclonal antibody (Low et al., 2001) and control rat IgG were covalently coupled to NHS-activated Sepharose 4B resin (Amersham). 1x10^10 BC3 cells were washed in PBS and incubated for 30 minutes at 4°C in 10 ml lysis buffer [20 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.2% NP-40, 10% glycerol, 1 mM PMSF and protease inhibitor cocktail (Roche)]. The lysate was centrifuged at 16,000 g for 10 minutes and then the cytoplasmic extract was divided equally between vFLIP and control resin for incubation at 4°C for 2 hours. The resin was washed three times in lysis buffer with 500 mM NaCl and 100 μl SDS-PAGE sample buffer lacking β-mercaptoethanol was added to elute immunoprecipitated proteins. The sample buffer was removed from the resin and β-mercaptoethanol was added and the samples were heated to 95°C for 4 minutes. The samples were divided 9:1 between two 12% SDS-PAGE gels. The gel containing 90% of the sample was stained with a Colloidal Blue Coomassie staining kit (Invitrogen). The gel containing 10% of the sample was stained using silver.

In-gel digest

Protein bands of interest were excised from the Coomassie stained gel and extracted with 200 mM ammonium bicarbonate / 50% acetonitrile, reduced with 20 mM DTT and then alkylated in 5 mM iodoacetamide and dehydrated. The gel slices were swollen in a minimal volume of 2 ng μl−1 trypsin (Promega) in 5 mM ammonium bicarbonate for in-gel digestion. Peptide mass fingerprinting was performed using a Reflex III time-of-flight mass spectrometer (Bruker Daltonik) with a nitrogen laser and a Scout-384 probe, to obtain positive ion mass spectra of digested protein with pulsed ion extraction in reflectron mode. An accelerating voltage of 26 kV was used with detector bias gating set to 2 kV and mass cut-off of m/z = 650. Matrix surfaces were prepared using recrystallised o-cyano-4-hydroxycinnamic acid and nitrocellulose using the fast evaporation method (Vorm et al., 1994). 0.4 μl of digestion supernatant was deposited on the matrix surface and allowed to dry prior to desalting with water. Peptide mass fingerprints thus obtained were searched against the non-redundant protein database of the National Centre for Biotechnology Information (NCBI) using the program MASCOT (Perkins et al., 1999).

Yeast two-hybrid interaction

Proteins interacting with vFLIP were identified using high throughput yeast-two-hybrid analysis at Hybrigenics (Paris). The VFLIP bait was constructed as a LexA, C-terminal fusion in the pB27 plasmid derived from the original pBTM116 (Vojtek et al., 1995). To generate an expression library, a randomly primed cDNA library from human placenta poly(A+) RNA was constructed and inserted into the pP6 plasmid derived from pACT2 (Rain et al., 2001). The library was then transformed into yeast and 10⁷ independent yeast colonies were collected, pooled and stored at −80°C in aliquots. The screen was performed to ensure that at least 5x10⁸ interactions were tested. The mating protocol has been described elsewhere (Fromont-Racine et al., 2002). The screening conditions were optimized for vFLIP bait using a test screen before performing the full-size screening. For all the selected clones, LacZ activity was measured in a semiquantitative X-Gal overlay assay. The prey fragments of the positive clones were amplified by PCR, analysed on agarose gel, and sequenced at their 5’ and 3’ junctions on a PE3700 sequencer. The resulting sequences were then used to identify the corresponding gene in the GenBank database (NCBI) using an automated Blast analysis procedure. Clones obtained many times in different screens against the same libraries were discounted as false positives.

Gel filtration

2x10⁷–2x10⁸ cells were incubated in lysis buffer (25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1 mM EGTA, 1 mM DTT, 0.2% NP-40, 5% glycerol, 1 mM Na3VO4, 10 mM β-glycerophosphate, 5 mM NaF, 1 mM PMSF and protease inhibitor cocktail) for 30 minutes at 4°C. The extract was centrifuged at 100,000 g for 1 hour at 4°C. 100 μl of the supernatant was loaded on a Superox 6 PC 3.2/30 column (Amersham) previously equilibrated in Buffer B (25 mM Tris-HCl pH 7.6, 150 mM NaCl, 0.2% NP-40, 5% glycerol). The fractionation was performed using an LKBμSeparation unit (Amersham) controlled using Smart Manager 5.1 software. The flow rate of the column was maintained at 40 μl min−1 and 22 fractions of 100 μl each were collected. 25 μl of each fraction were separated by SDS-PAGE gel for immunoblot, whereas 50 μl of each fraction was used for kinase assays. The column was calibrated in Buffer B using protein standards: thyroglobulin (669 kDa), ferritin (440 kDa) and catalase (232 kDa) (Amersham).

Small-scale immunoprecipitation, GST pull down and immunoblotting

Cytoplasmic extracts from transfected 293T cells were incubated either with 1.5 μg of vFLIP antibody and 20 μl protein-G/Sepharose...
protease inhibitor cocktail (Roche). Proteins were separated by electrophoresis on a 12% SDS-polyacrylamide gel then transferred to Hybond ECL nitrocellulose membranes (Amersham) for immunoblot analysis. Blots were incubated overnight at 4°C in blocking solution (PBS containing 5% low-fat milk and 0.1% Tween 20) and then incubated with primary antibody for 1 hour. Primary antibodies: anti-vFLIP 6/14 antibody (1:100 dilution), anti-Xpress (1:5000 dilution) incubated with primary antibody for 1 hour. Primary antibodies: anti-vFLIP 6/14 antibody (1:100 dilution), anti-Xpress (1:5000 dilution) (Invitrogen 46-0528), anti-IKK (Invitrogen 46-0528), anti-IKK antibody and protein-G/Sepharose. For kinase assays using anti-IKK antibody, an additional pre-clearance step of 1 hour at 4°C with 1.5 μg normal rabbit serum and 20 μl protein-G/Sepharose was included. Immune complexes were washed three times in 0.5 ml high salt buffer (25 mM Tris-HCl pH 7.6, 500 mM NaCl, 1 mM EGTA, 1 mM DTT, 0.2% NP-40, 5% glycerol, 1 mM Na3VO4, 10 mM β-glycerophosphate, 5 mM NaF, 1 mM PMSF and protease inhibitor cocktail) (gift from S. Mittnacht, Institute of Cancer Research, London, UK). Bound antibodies were detected with peroxidase-conjugated secondary antibodies (1:2000 dilution) and visualized using electrochemical luminescence (ECL) (Amersham).

IkBα kinase assay

For kinase assays, 50 μl of each column fraction or 100-200 μg of cytoplasmic extract were incubated for 2 hours at 4°C with 1.5 μg antibody and protein-G-Sepharose. For kinase assays using anti-IKK antibody, an additional pre-clearance step of 1 hour at 4°C with 1.5 μg normal rabbit serum and 20 μl protein-G/Sepharose was included. Immune complexes were washed three times in 0.5 ml high salt buffer (25 mM Tris-HCl pH 7.6, 500 mM NaCl, 1 mM EGTA, 1 mM DTT, 0.2% NP-40, 5% glycerol, 1 mM Na3VO4, 10 mM β-glycerophosphate, 5 mM NaF, 1 mM PMSF and protease inhibitor cocktail). Immune complexes were then washed a further two times in kinase wash buffer (20 mM HEPES pH 7.6, 50 mM NaCl, 20 mM β-glycerophosphate, 0.5 mM DTT, 1 mM PMSF) before 40 μl kinase reaction buffer (20 mM HEPES pH 7.6, 50 mM NaCl, 10 mM MgCl2, 2 mM DTT, 20 μM ATP, 0.1 mM Na3VO4 and protease inhibitor cocktail) was added. 0.5 μl of F35-γ-ATP and 1 μg of wild-type IkBα or mutant IkBα truncated at positions 1-54 (S32A/S36A) GST fusion protein was added to each reaction, which were incubated at 30°C for 30 minutes and then stopped by the addition of SDS-PAGE sample buffer. The samples were separated by 12% SDS-PAGE and radiolabelled phosphoproteins were visualized by autoradiography.

Cell viability assays

The viability of BC3 populations was measured directly by haemocytometry. For annexin-V-propidium-iodide binding assays, 105 cells were washed once in cold PBS before staining with TACS™ Annexin-V-FITC Apoptosis detection kit (R&D Systems) and analysis with a FACSCaliber using CellQuest software (Becton Dickinson, Franklin Lakes, NJ).
Results

vFLIP interacts with the γ subunit of the IKK complex

A yeast-two-hybrid screen was performed to identify proteins capable of interacting with vFLIP. 14 IKKγ clones were identified, including six independent fusions (Fig. 1B). This suggests a direct interaction between vFLIP and IKKγ, because the IKK complex has not been described in yeast (Epinat et al., 1997). The minimum common sequence between the six independent fusions suggested that the domain in IKKγ required for contact with vFLIP is between amino acids 173-272, in the third coiled-coil region (CCR3) and first section of CCR4 (Fig. 1A,B). IKKγ mutants (Fig. 1C) were therefore constructed with an N-terminal Xpress tag and co-transfected with or without vFLIP in 293T cells. Fig. 1D shows that all IKKγ fragments, with the exception of amino acids 1-150, co-immunoprecipitated with vFLIP, which mapped the minimum interacting domain to amino acids 150-272 of IKKγ. However, the level of vFLIP in the cells expressing amino acids 1-150 of IKKγ was consistently lower (Fig. 1D and data not shown), perhaps because interaction with IKKγ stabilized vFLIP. We therefore made the same mutants as GST fusion proteins and examined their ability to bind vFLIP in lysate from transfected 293T cells. Fig. 1E shows that all GST-IKKγ fragments with the exception of amino acids 1-150 bound vFLIP, confirming the minimum vFLIP interacting domain as amino acids 150-272 of IKKγ.

Endogenous vFLIP is associated with an activated IKK complex

To identify proteins interacting with the endogenous vFLIP in cells infected with KSHV, we purified vFLIP from BC3 cells by immunoprecipitation. Proteins that co-immunoprecipitated with vFLIP but were not precipitated by a control rat antibody were excised from the Coomassie stained gel and identified by mass spectrometry as indicated.
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Fig. 3A shows that all the soluble vFLIP in BC3 cells is present in a high molecular weight protein complex. The three components of the IKK complex (IKKa, IKKβ and IKKγ) eluted from the Superose 6 column in the same fractions as vFLIP. The Superose 6 fractions were also analysed for IkBα kinase activity associated with vFLIP (Fig. 3A, bottom). Kinase activity was found in fractions 4-7, with the major peak in fraction 5, identical to the distribution of vFLIP and IKK.

Fig. 3B demonstrates the specificity of the kinase assay. Immune complexes precipitated using an isotype-matched control antibody did not have an associated kinase activity and the vFLIP immune complex was not able to phosphorylate a mutant GST-IκBα containing point mutations at the two IKK targets in IκBα, S32A and S36A. Fig. 3C shows that all detectable vFLIP in BC3 cell lysate is associated with IKKγ, because immunoprecipitation with an anti-IKKγ antibody depleted vFLIP from cell lysate, but did not affect IκBα.

Activity of the vFLIP-IKK complex depends upon Hsp90
In KSHV-infected B cells, other viral or cellular proteins might co-operate with vFLIP to activate IKK. To investigate whether vFLIP expressed at a similar level to that in BC3 cells was sufficient to activate the IKK complex, we transduced 293T cells with a lentiviral vector encoding either vFLIP plus GFP (A,B) or GFP alone (C) and then treated with DMSO or geldanamycin (GA), as indicated, was subjected to gel filtration on a Superose 6 column. Fractions were analysed by immunoblot with anti-vFLIP or anti-IKKγ antibodies; in A and B fractions were also immunoprecipitated with the anti-vFLIP antibody 6/14 and IκBα kinase activity was measured.

Fig. 4. The vFLIP-IKK kinase activity is inhibited by geldanamycin. Cytoplasmic extract from 293T cells transduced with a lentivirus encoding either vFLIP plus GFP (A,B) or GFP alone (C) and then treated with DMSO or geldanamycin (GA), as indicated, was subjected to gel filtration on a Superose 6 column. Fractions were analysed by immunoblot with anti-vFLIP or anti-IKKγ antibodies; in A and B fractions were also immunoprecipitated with the anti-vFLIP antibody 6/14 and IκBα kinase activity was measured.

To investigate the role of Hsp90 in the vFLIP-IKK complex, we used the nucleotide analogue GA, which inhibits the function of Hsp90 (Whitesell et al., 1994). We found no change in the size of the vFLIP-IKK complex (Fig. 4B) or the inactive IKK complex in control 293T cells (Fig. 4C), or on the levels of IKKa, IKKβ or IKKγ expression (data not shown) upon GA treatment. We did observe vFLIP in lower fractions in both control and GA-treated cells, and attribute this to vFLIP being in excess of the IKK components. However, IKK activity associated with vFLIP in GA treated cells was significantly reduced (Fig. 4B). The activity of the vFLIP-IKK complex is therefore dependent on Hsp90.

GA kills PEL cells
We then examined whether GA could inhibit IKK activity and cause death of KSHV-infected BC3 cells. Fig. 5A shows that 0.5 μM GA inhibited activity of the vFLIP-IKK complex in BC3 cells. This concentration of GA also caused a loss in viability of BC3 cells: after 48 hours, 72% of GA-treated cells were dead, compared with 35% of the control BC3 cells treated with DMSO in serum-free medium (Fig. 5B). Cell death induced by GA might be either apoptosis or necrosis, because the dying cells stained with Annexin-V (Koopman et al., 1994), which identifies cells that have lost phosphatidylserine polarity in the plasma membrane, and with propidium iodide, which detects loss in plasma membrane integrity (Fig. 5C,D).

Discussion
This study focuses on the role of KSHV vFLIP in latently infected PEL cells. Chaudhary and co-workers have demonstrated that vFLIP associates with the IKK complex and activates NF-κB reporter constructs when expressed in non-lymphoid cells (Chaudhary et al., 1999; Liu et al., 2002). Our data support their observations and extends their conclusion to a KSHV-infected PEL cell line. We used a rat monoclonal antibody to immunoprecipitate vFLIP from a PEL cell line (BC3) and demonstrated that vFLIP protein associates with and activates the IKK complex. All the soluble vFLIP in these cells co-elutes with active IKK on a gel filtration column and co-
precipitates with IKKγ. vFLIP might activate IKK by recruitment of an upstream activator such as a member of the mitogen-activated protein kinase kinase kinase family (Karin et al., 2000). However, IKKα and IKKβ can autophosphorylate the IKK activation loop when overexpressed in mammalian cells (Woronicz et al., 1997). vFLIP might therefore induce a conformational change in the complex, inducing autophosphorylation and autoactivation.

We also demonstrated that vFLIP directly contacts IKKγ, which is analogous to the function of the Tax protein of human T-cell leukaemia virus type 1 (HTLV-1). Transformation of T cells by HTLV-1 is mediated by the regulatory protein Tax, which stimulates expression of various genes regulated by NF-κB (Sun and Ballard, 1999). Tax has been shown to stimulate IKK activity (Chu et al., 1998; Geleziunas et al., 1998; Uhlik et al., 1998; Yin et al., 1998) by binding directly to IKKγ (Chu et al., 1999; Harhaj et al., 1999; Jin et al., 1999; Xiao et al., 2000). IKKγ is predicted to contain five major coiled-coil domains (Rothwarf et al., 1998; Sun et al., 2000), of which the second and fifth from the N-terminus contain leucine zipper motifs (LZ1 and LZ2, respectively) (Fig. 1). Deletions of LZ1 abolish the binding of Tax to IKKγ, whereas mutants lacking LZ2 show reduced Tax-IKKγ interaction (Xiao et al., 2001). By contrast, our data demonstrate that a region of IKKγ including CCR3 and CCR4, between amino acids 150 and 272, is crucial for vFLIP interaction. This shows that the structurally unrelated viral proteins Tax and vFLIP have evolved distinct mechanisms to bind IKKγ and thereby activate IKK.

Constitutive activation of NF-κB is a common feature of viruses that transform lymphoid cells. Among the gammaherpesviruses, the latent membrane protein 1 (LMP-1) of Epstein-Barr virus activates the NF-κB pathway by TRADD and TRAF recruitment to its cytoplasmic tail (Farrell, 1998). K15, the LMP-1 homologue encoded by KSHV, can interact with TRAFs (Glenn et al., 1999) but its role in NF-κB activation in KSHV-infected cells remains unclear. Orf74 of KSHV encodes a constitutively active chemokine receptor homologue that activates NF-κB (Schwarz et al., 2001) but Orf74 is not latently expressed in KSHV-infected PEL cells (Chiu et al., 2002). However, the K1 transmembrane protein is expressed in PEL cells and has been implicated in NF-κB activation by transgenic mouse experiments (Prakash et al., 2002). KSHV might therefore use multiple, possibly cooperative, strategies to activate NF-κB in different target cells and at various points in the viral life cycle. It is intriguing that a third gammaherpesvirus, herpesvirus saimiri, activates NF-κB by co-operative action of two saimiri-specific transforming proteins, Tip and StpC (Lee et al., 1999; Merlo et al., 2001; Yoon et al., 1997).

In addition to the IKK subunits, we found Hsp90 associated with vFLIP in BC3 cells. This is consistent with a previous report that Hsp90 and a co-chaperone, Cdc37, are additional components of the IKK complex (Chen et al., 2002). This previous study demonstrated that the Hsp90 inhibitor GA prevented both TNF-induced membrane recruitment of the IKK complex to TNF-R1 and TNF-induced IKK activation (Chen et al., 2002). GA also inhibited activity of the vFLIP-IKK complex, although we did not observe the dissociation of IKKγ from the IKK complex reported by Chen et al. (Chen et al., 2002). Consistent with the inhibition of vFLIP-IKK activity, GA also induced death of BC3 cells. This suggests that vFLIP activation of IKK is crucial in the maintenance of BC3 cell survival. vFLIP activation of the NF-κB pathway has also been shown to inhibit apoptosis when vFLIP was ectopically expressed in a human leukaemic cell line (Sun et al., 2003). GA analogues are promising anticancer agents because Hsp90 is crucial for maintaining the function of several oncogenic proteins (Maloney et al., 2002). Our data suggest that they
might also be effective in the treatment of KSHV-related malignancies.

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