Functions of KSHV-encoded FLIP

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Abstract

Kaposi's sarcoma (KS)-associated herpesvirus (KSHV) was discovered in 1994. KSHV is associated with at least three types of human cancer; KS, an endothelial tumour, and two lymphoproliferative disorders, primary effusion lymphoma (PEL) and a variant of multicentric Castleman's disease (MCD). In KSHV, the genes expressed in latency have been implicated in cell transformation. One of a cluster of three latency-associated genes, that regulate proliferation and apoptosis, encodes a viral FLICE inhibitory protein (vFLIP) in open reading frame 71 (ORF71). Two roles have been proposed for vFLIP; when expressed in heterologous cells it both blocks Fas-mediated apoptosis and activates the NF-κB pathway by interaction with IκB kinase (IKK). Given the two contrasting roles assigned to vFLIP, the aim of this study was to determine the function of vFLIP in KSHV-infected cells. vFLIP was therefore immunoprecipitated from PEL cells and four associated proteins were identified by mass spectrometry: IKK components IKKα, β, γ and the chaperone, Hsp90. Using gel filtration, 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 vFLIP-induced IKK activity and killed PEL cells, inferring that vFLIP activation of NF-κB contributes to PEL survival. In a yeast-two-hybrid screen, our collaborators identified IKKγ as an interacting partner of vFLIP. Fragments of IKKγ were expressed in mammalian cells and bacteria, and the central portion of IKKγ (amino acids 150-272) was identified as the vFLIP binding region. Finally, it is suggested that vFLIP activates the alternative pathway of NF-κB activation, leading to processing of p100 and generation of p52. This process is phosphorylation dependent and results in nuclear translocation of RelB and p52. A possible mechanism of action is suggested by the physical interaction between p100/p52 and vFLIP. These data strongly support an important role for vFLIP in NF-κB activation. This may be crucial for cell transformation by KSHV, for survival of infected cells and for the maintenance of latency.

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Finally, thanks to my parents and sister.

To the memory of Edward Pepper and Cicely Field,

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Abbreviations

AHV	alcelaphine herpesvirus
AIDS	acquired immuno deficiency syndrome
Aly	alymphoplasia
AP-1	activating protein-1
Apaf-1	apoptotic-protease activating factor-1
ATF-4	activating transcription factor-4
ATL	adult T cell leukaemia
ATP	adenosine triphosphate
BAFF	B cell activating factor
Bak	Bcl-2 antagonist/killer
Bax	Bcl-2 associated X protein
BCBL	body cavity based lymphoma
Bcl	B cell lymphoma
bFGF	basic fibroblast growth factor
BLC	B lymphocyte chemoattractant
BoHV	bovine herpesvirus
bp	base pair
BR3	BAFF receptor 3
βTrCP	β-transducing repeat-containing protein
CAML	calcium-modulating cyclophilin ligand
CAMP	cyclic AMP
CARD	caspase recruitment domain
CBP	CREB-binding protein
CCR	coiled-coil region
CDC	cell division cycle
cdk	cyclin-dependent kinase
$cFLIP_{L/S}$	cellular FLIP long/short
CHUK	conserved helix-loop-helix ubiquitous kinase
cIL-6	cellular IL-6
CREB	cAMP-response element-binding protein
CTAR	C-terminal activating region
C-terminus	carboxy-terminus
CTL	cytotoxic T lymphocyte
DED	death effector domain
DD	death domain
DISC	death inducing signalling complex
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate mix
DR	death receptor
ds	double strand
DTT	dithiothreitol
DUB	de-ubiquitinating
EBNA	Epstein-Barr nuclear antigen
EBV	Epstein-Barr virus
EDA-R	ectodermal dysplasia receptor
EDTA	ethylene-diamine-tetra-acetic acid
eGFP	enhanced green fluorescent protein
EHV	equine herpesvirus
ELC	Ebl-1-ligand chemokine
EMCV	encephalomyocarditis virus
EMSA	electrophoretic mobility shift assay

Env	HIV envelope
E-selectin	endothelial-leukocyte adhesion molecule-selectin
EST	expressed sequence tag
FACS	fluorescent-activated cell sorter
FADD	Fas-associated death domain
FCS	foetal calf serum
Fig.	figure
FLICE	FADD-like-ICE
FLIP	FLICE-inhibitory protein
g	relative centrifugal force
gag	HIV group associated antigen
GRR	glycine rich region
GST	glutathione S-transferase
h	hours
HA	haemagglutinin
HAART	highly active anti-retroviral therapy
HAT	histone acetylase
HCMV	human cytomegalovirus
H-DNA	high GC content DNA
HED-ID	hypohidrotic ectodermal dysplasia with immune deficiency
HEK	human embryonic kidney
HHV	human herpesvirus
HIV	human immunodeficiency virus
HMG I(Y)	high mobility group I(Y)
HPV	human papilloma virus
Hsp90	heat shock protein 90
HSV	herpes simplex virus
HTLV-1	human T cell leukaemia virus -1
HUVEC	human umbilical vein endothelial cell
HVA	herpesvirus ateles
HVS	herpesvirus saimiri
IAP	inhibitor of apoptosis
ICAM	intercellular adhesion molecule
ICE	IL-1β converting enzyme
IFA	immunofluorescence assay
IFN	interferon
Ig	immunoglobulin
IKAP-1	IKK associated protein-1
IκB	inhibitor of κB
IKK	IκB kinase
IL	interleukin
IP	incontinentia pigmenti
IPTG	isopropylthio-β-D-galactoside
IRES	internal ribosome entry site
IRF	IFN regulatory factor
ISH	*in situ* hybridisation
ISS	immunostimulatory sequences
ITAM	immuno receptor tyrosin-based motif
JNK	jun N-terminal kinase
Kb	kilobase
KDa	kilodalton
KS	Kaposi's sarcoma
KSHV	KS-associated herpesvirus
LANA	latent nuclear antigen

LB	Luria-Bertani
L-DNA	low GC content DNA
LMP	latent membrane protein
LPS	lipopolysaccharide
LT	latent transcript
LTβR	lymphotoxin β receptor
LTR	long terminal repeat
LZ	leucine zipper
MACH	MORT1-associated ced3 homologue
MALT	mucose associated lymphoid tissue
MAPK	mitogen activated protein kinase
MCD	multiple Castleman's disease
MCV	molluscum contagiosum virus
MEF	mouse embryo fibroblast
MEKK	MAP/Erk kinase kinase
MHV	murine herpesvirus
min	minutes
MIR	modulator of immune recognition
MOI	multiplicity of infection
MPC	MALT1/paracaspase
mRNA	messenger RNA
nef	HIV negative regulatory factor
NEMO	NF-κB essential factor
NFAT	nuclear factor of T cells
NF-κB	nuclear factor of κ light polypeptide gene enhancer in B cells
NGF-R	nerve growth factor receptor
NIK	NF-κB inducing kinase
NLS	nuclear localisation signal
NP40	nonidet P-40
N-terminus	amino-terminus
OD	optical density
ORC	origin recognition complex
ORF	open reading frame
PAGE	polyacrylamide electrophoresis gel
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEL	primary effusion lymphoma
PHA	phytohaemagglutinin
PI	propidium iodide
PID	processing inhibitory domain
PI-3K	phosphatidylinositol 3-kinase
PIM	protein inhibitor mix
PKC	protein kinase C
PKR	dsRNA-activated serine-threonine protein kinase
PMA	phorbal myristate acetate
PNK	polynucleotide kinase
pol	HIV polymerase
PP2A	protein phosphatase 2A
pRb	retinoblastoma protein
PRD	positive regulatory domain
PS	phosphatidyl serine
RAFTK	related adhesion focal tyrosine kinase
RANK	receptor activator of NF-κB
Rel	reticuloendotheliosis

rev	HIV regulator of virion protein expression
RHD	rel homology domain
RIP	receptor interacting protein
RIPA	radioimmunoprecipitation lysis buffer
RNA	ribonucleic acid
RNAi	RNA-interference
RPMI	Roswell Park Memorial Institute 1640 medium
RRV	rhesus rhadinovirus
s	seconds
SAP	stress activated protein
SCF	SKP1 cullin F box
SDS	sodium dodecyl citrate
SEM	standard error of the mean
SH	src homology
SLC	secondary lymphoid tissue chemokine
STP	saimiri transforming protein
SUMO	small ubiquitin-like modifier
SV40	simian virus 40
TAB	TAK1 binding protein
TAE	tris-acetate-EDTA
TAK1	TGFβ-activated kinase-1
tat	HIV transactivator of transcription
TBE	tris-borate-EDTA
TCR	T cell receptor
TGFβ	transforming growth factor-β
Th	T helper lymphocyte
Tip	tyrosine kinase interacting protein
TNF	tumour necrosis factor
TNF-R	TNF-receptor
TPA	tetradecanoyl phorbal acetate
TRADD	TNFR-associated death domain
TRAF	TNF-associated factor
TRAIL	TNF-related apoptosis-inducing ligand receptor
TRAMP	TNF-related apoptosis-mediating protein
TRIKA	TRAF6-regulated IKK activator
TWEAK	TNF-like weak inducer of apoptosis
VCAM	vascular cell adhesion molecule
vCBP	viral complement binding protein
vCLAP	viral CARD-like apoptotic protein
vcyclin	viral cyclin
VEGF	vascular endothelial growth factor
vFLIP	viral FLIP
vGPCR	viral G-protein coupled receptor
vif	HIV virion infectivity factor
vMIP	viral macrophage inflammatory protein
vpr	HIV viral protein R
vpu	HIV viral protein U

CHAPTER 1

Introduction

This study concerns the function of Kaposi's sarcoma-associated herpesvirus (KSHV) vFLIP (viral Fas-associated death domain [FADD]-like IL-1 converting enzyme {FLICE}-inhibitory protein). This protein was considered to block apoptosis directly, but has also been shown to activate the transcription factor NF-κB. vFLIP is a candidate-transforming factor for KSHV. The first section of this chapter will review the biology of KSHV, concentrating on other KSHV genes that have important roles in viral pathogenesis and oncogenesis. The second section will deal with the NF-κB pathway and its regulation. These sections provide the context for an account of vFLIP function, which comprises the third and final section.

1.2 Kaposi's sarcoma-associated herpesvirus (KSHV)

1.2.1 Discovery of KSHV

In 1981, a highly aggressive form of Kaposi's sarcoma (KS) was identified as part of the new AIDS epidemic and was termed "epidemic KS" (Borkovic et al, 1981; Gottlieb et al, 1981). Previously a rare disorder, the Hungarian dermatologist Moritz Kaposi first described the angiomatous neoplasm affecting elderly men of Mediterranean or Jewish descent that later became known as "classical KS" in 1872 (Kaposi 1872). Two other epidemiological forms of KS are recognised: endemic KS in equatorial Africa and iatrogenic or post-transplant KS. The AIDS epidemic drew attention to KS and epidemiological studies of human immunodeficiency virus (HIV)-infected populations revealed a transmissible agent as the most likely cause of KS (Weiss et al, 1986; Beral 1991). The breakthrough in confirming the infectious nature of KS came in 1994, when Chang and co-workers used representational difference analysis to identify two DNA fragments that were uniquely present in the diseased tissue of an AIDS-KS patient (Chang et al, 1994). The 330 and 631 bp fragments were found to have significant amino acid identity to the capsid and tegument proteins of two gammaherpesviruses, Epstein-Barr virus (EBV) and herpesvirus saimiri (HVS), both capable of cell transformation. Soon after, DNA belonging to the novel KS-associated herpesvirus (KSHV) was detected in cells derived from patients with primary effusion lymphoma (PEL), a rare lymphoma of B cells normally associated with AIDS (Cesarman et al, 1995a; Cesarman et al, 1995b). PEL cells support continuous KSHV infection in culture and provided a vital research tool (Moore et al, 1996b), enabling nucleotide sequencing (Russo et al, 1996) and visualisation of herpesvirus-like KSHV virions by electron microscopy (Said et al, 1996; Orenstein et al, 1997). The near complete KSHV genome was also sequenced from a KS biopsy (Neipel et al, 1998). Together, these data confirmed the classification of KSHV as the eighth human herpesvirus (HHV-8).

1.2.2 Viral taxonomy

Herpesvirus taxonomy was traditionally based upon virion structure and cell tropism, but is now largely determined by genomic structure and sequence (Davison 2002). Using these criteria, KSHV has been assigned membership of the γ-herpesvirus sub-family. The γ-herpesviruses are further divided into two genera: the γ-1 or lymphocrytoviruses of which EBV is the prototype member and the γ-2 or rhadinoviruses of which HVS is the classic prototype (Fickenscher et al, 2001; Moore et al, 2001). An artist's impression of the herpesvirus evolutionary tree is shown in Fig. 1.1. KSHV is currently the only human member of the rhadinovirus genus, its closest human relative being EBV (McGeoch et al, 1999; Montague et al, 2000; Alba et al, 2001). The human herpesviruses are listed in Table 1.1.

1.2.3 Virion structure

The herpesvirus virion is characteristically large, 200-250 nm in diameter, with a thick-walled nucleocapsid that is surrounded by a proteinaceous layer called the tegument (Steven et al, 1997). In turn, the tegument is surrounded by a lipid envelope bilayer that is derived from the host cell membrane and is studded with viral glycoproteins (Rixon 1993; Gibson 1996; Homa et al, 1997). The genome of the virus is packaged as linearised double-stranded DNA at the core of this structure in liquid-crystalline form (Booy et al, 1991). The three-dimensional structure of the KSHV capsid has been solved by computer reconstruction of cryogenic electron microscope images to reveal an icosahedral lattice composed of pentons, hexons and triplexes (Wu et al, 2000).

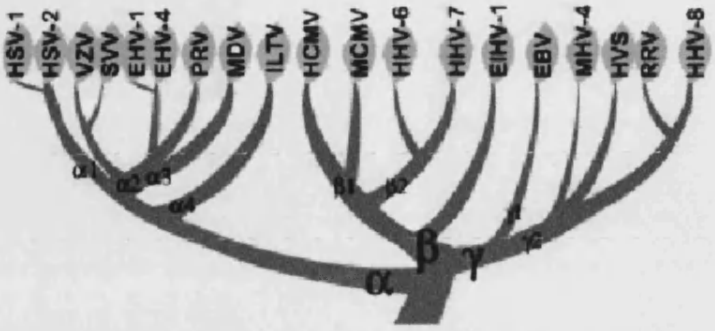


Figure 1.1. Herpesvirus evolutionary tree.

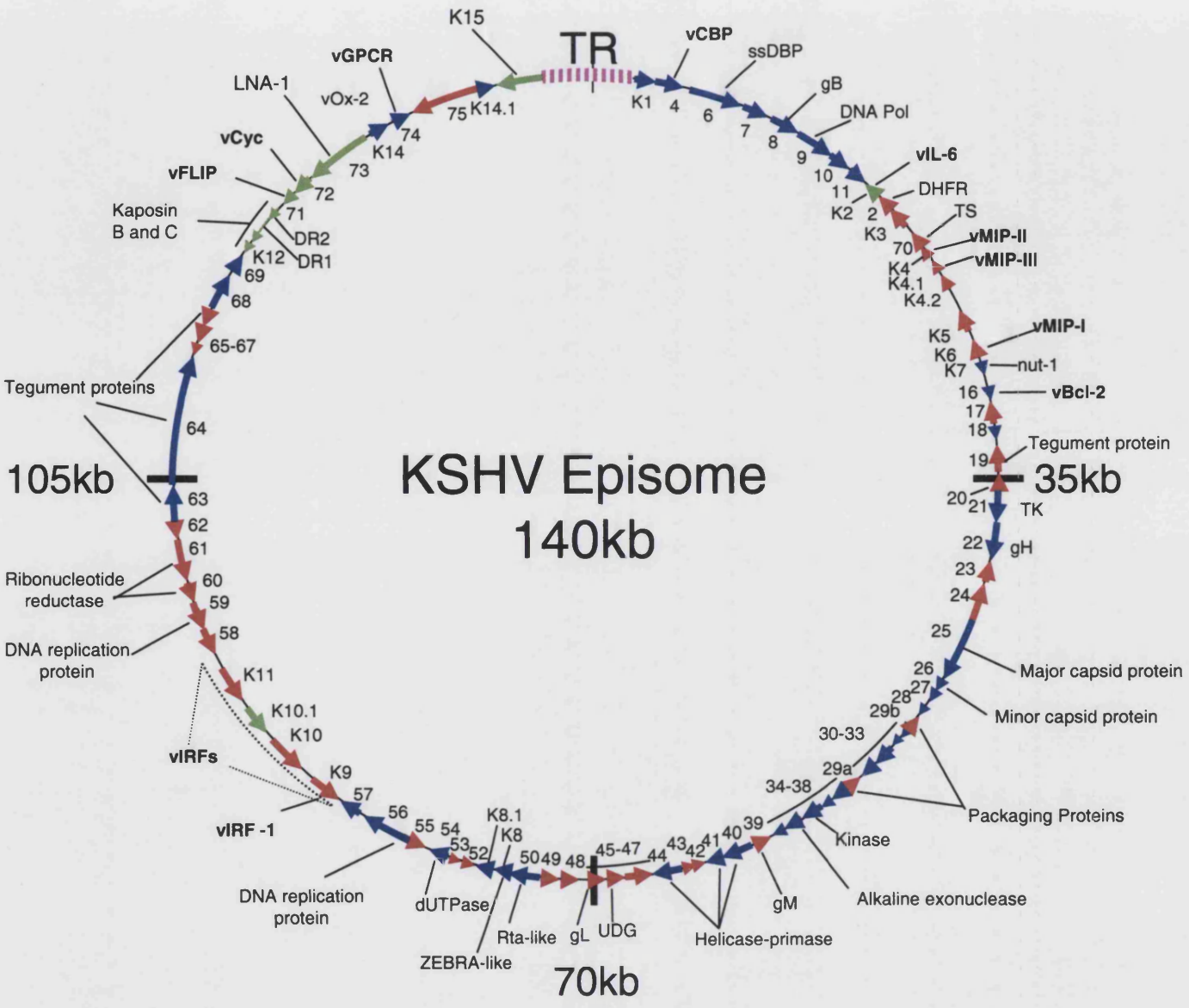
An artist's impression of the herpesvirus evolutionary tree (Davison, 2002). Each branch leads to a herpesvirus subfamily (α, β and γ) and from thence to genera (α1, α2, etc.). Individual examples are given for each genera, including each of the human herpesviruses, which are listed in Table 1.1 below. Abbreviations for the non-human herpesviruses include SVV, simian varicella virus; EHV-1, -4, equine herpesvirus; PRV, pseudorabiesvirus; MDV, Marek's disease virus; ILTV, infectious laryngotracheitis virus; MCMV, murine cytomegalovirus; E1HV-1, elephant endotheliotropic herpesvirus; MHV4, murine herpesvirus 68; HVS, herpesvirus saimiri; RRV, rhesus rhadinovirus.

Common name	Human herpesvirus	Disease associations
Herpes-simplex virus-1 (HSV-1)	HHV-1	Oropharangeal herpes Genital herpes
Herpes-simplex virus-2 (HSV-2)	HHV-2	Genital herpes
Varicella-zoster virus (VZV)	HHV-3	Varicella (chicken pox) Zoster (shingles)
Epstein-Barr virus (EBV)	HHV-4	Infectious mononucleosis Nasopharyngeal carcinoma Post-transplant lymphoproliferative disorder AIDS-related lymphoma Burkitt's lymphoma Hodgkin's disease
Human cytomegalovirus (HCMV)	HHV-5	CMV-mononucleosis CMV retinitis Cytomegalic inclusion disease of the newborn
Human herpesvirus-6	HHV-6	Exanthem subitum (6^{th} disease)
Human herpesvirus-7	HHV-7	Exanthem subitum (6^{th} disease)
Kaposi's sarcoma-associated herpesvirus (KSHV)	HHV-8	Kaposi's sarcoma Primary effusion lymphoma Multicentric Castleman's disease

Table 1.1. Human herpesviruses and their disease associations.

1.2.4 Genomic organisation

The KSHV genome consists of a long central portion of coding DNA with low GC content (L-DNA) flanked by multiple terminal repeats with high GC content in excess of 84% (H-DNA) (Russo et al, 1996; Schulz 1998). Similar to other rhadinoviruses, the L-DNA of KSHV comprises of 140.5 kb of "unique" DNA containing at least 85 open reading frames (ORFs) (Russo et al, 1996; Moore et al, 1996b; Neipel et al, 1997). The coding DNA is flanked by two 801 bp terminal repeats (Russo et al, 1996), to give a total size estimated by Gardella gel of 170 kb (Renne et al, 1996a). The genome bears remarkable similarity to that of HVS, and the nomenclature of KSHV genes is derived from HVS, as the prototype rhadinovirus. The two viruses share 66 homologous genes upon which this nomenclature is based. Within KSHV, these genes are numbered consecutively from left to right across the genome and given the prefix "ORF". Interspaced within this structure are genes originally thought to be unique to KSHV, designated K1 to K15. However, K3 (MIR1), K5 (MIR2), K7 (vIAP) and K13 (vFLIP) have subsequently been found to have homologues and some additional unique genes have been added (including K4.1, K4.2, K8.1, K10.1, K10.5, K11.1, K14.1). Approximately half of the genes encoded by KSHV have now been ascribed a function, largely on the basis of sequence similarity to genes of known function (Jenner et al, 2002; Holzerlandt et al, 2002). Amongst these genes are a striking number that have been pirated from the host, including viral homologues of interleukin-6, Bcl-2, cyclin D, a G protein-coupled receptor and cFLIP. It has been proposed that many of these "pirated" genes were acquired because they allow the virus to directly manipulate the host cellular machinery (Neipel et al, 1997; Moore et al, 1998; Choi et al, 2001). The structure of the KSHV episome is depicted in Fig. 1.2.


KSHV Episome
140kb
105kb
35kb
70kb
TR
K15
vGPCR
LNA-1
vOx-2
vCyc
vFLIP
Kaposin
B and C
DR2
DR1
K12
K14
K14.1
75
74
73
72
71
69
68
65-67
64
63
62
61
60
59
58
K11
K10.1
K10
K9
57
56
55
54
K8.1
K8
53
52
50
49
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45-47
44
43
42
41
40
39
34-38
30-33
29a
29b
28
27
26
25
24
23
22
21
20
19
18
17
16
K7
K6
K5
K4.2
K4.1
K4
70
K3
K2
11
10
9
8
7
6
4
K1
vCBP
ssDBP
gB
DNA Pol
vIL-6
DHFR
TS
vMIP-II
vMIP-III
vMIP-I
nut-1
vBcl-2
Tegument protein
TK
gH
Major capsid protein
Minor capsid protein
Packaging Proteins
Kinase
Alkaline exonuclease
gM
Helicase-primase
UDG
gL
Rta-like
ZEBRA-like
dUTPase
DNA replication
protein
vIRF -1
vIRFs
DNA replication
protein
Ribonucleotide
reductase
Tegument proteins

Figure 1.2. Structure of the KSHV episome.

Numbers outside of the episome represent nucleotide base pairs in kilobases (kb). Numbers within the episome indicate KSHV-encoded ORFs. Novel ORFs not present in other herpesviruses were designated K1 to K15. ORFs encoded on the forward strand are shown as arrows pointing clockwise, anticlockwise arrows indicate ORFs encoded on the reverse strand. Annotations outside the episome indicate putative function for each ORF, genes with cellular homologues are shown in bold. TR, terminal repeat; vCBP, viral complement binding protein; ssDBP, single-stranded DNA binding protein; gB, glycoprotein B; DNA Pol, DNA polymerase; vIL-6, viral interleukin-6; DHFR, dihydrofolate reductase; vMIP, viral macrophage inflammatory protein; nut-1 nuclear tRNA-like transcript; vBcl-2, viral B cell leukaemia-2; TK, thymidine kinase; TS, thymidine synthase; gH, glycoprotein H; gM, glycoprotein M; UDG, uracil DNA glucosidase; gL, glycoprotein L; vFLIP, viral FLIP; vcyc, viral cyclin; cGPCR, viral G-protein-coupled receptor. (taken from Sharp and Boshoff, 2000)

1.2.5 Diseases associated with KSHV

1.2.5.1 Kaposi's sarcoma

KS is a multi-centric lesion, characterised by three stages. The initial lesion (patch stage) is composed of granulation-like tissue with an inflammatory cell infiltrate of T cells, monocyte-macrophages and dendritic cells (Regezi et al, 1993; MacPhail et al, 1996; Fiorelli et al, 1998). These cells produce Th-1 type cytokines. As explained below, these cytokines are thought to activate endothelial cells to acquire the characteristic KS 'spindle cell' phenotype (Miles et al, 1990; Sturzl et al, 1995; Sirianni et al, 1998; Fiorelli et al, 1998). As the lesion develops to plaque stage, the initially sparse spindle cells expand throughout the dermis and create irregular vascular channels containing red blood cells. Spindle cells arranged in sheets predominate in the final lesions (nodular stage) and are considered to be the transformed cell type (Boshoff et al, 2001).

The precise origin of spindle cells is not known. The majority express endothelial markers, such as CD31 and CD34 (Weich et al, 1991; Sturzl et al, 1992). Many also express markers suggesting lymphatic origin, including vascular endothelial growth factor (VEGF-3) receptor-3 and podoplanin (Jussila et al, 1998; Dupin et al, 1999; Weninger et al, 1999). However, some cells are more characteristic of smooth muscle, macrophages and dendritic cells (Nickoloff et al, 1989; Sturzl et al, 1992; Uccini et al, 1994). These data, and in particular the extensive expression of VEGF-3, suggest that KS spindle cells probably belong to an endothelial precursor that can differentiate into lymphatic cells. Interestingly, although KSHV can transform primary human endothelial cells *in vitro* (Flore et al, 1998), KSHV genomes were not found in every transformed cell. A paracrine contribution, in which viral gene products and cytokines expressed in one cell can affect the growth characteristics of neighbouring cells, is therefore thought to be important in spindle cell formation and tumourigenesis (Dupin et al, 1999). Both spindle cells and the infiltrating inflammatory cells express high levels of cellular IL-6 (cIL-6), basic fibroblast growth factor (bFGF), VEGF, IL-1β, TNFα and IFNγ (Salahuddin et al, 1988; Miles et al, 1990; Fiorelli et al, 1998). cIL-6 promotes growth of KS cells *in vitro* (Miles et al, 1990), and IFNγ induces a spindle

cell-like phenotype in endothelial cells (Fiorelli et al, 1998) and also reactivates latent virus (Chang et al, 2000). VEGF functions in synergy with bFGF as a KS cell growth factor, enhancing the development of KS-like lesions when human AIDS-KS cells were injected into mice (Ensoli et al, 1989).

Only 10% of spindle cells are KSHV positive in the early patch lesions (Dupin et al, 1999), but the vast majority of spindle cells are KSHV-infected in late stage nodular tumours (Boshoff et al, 1995; Staskus et al, 1997; Sturzl et al, 1997; Dupin et al, 1999). These data infer that, like many tumours, KS begins as a polyclonal hyperplasia in which infected cells have a growth advantage. This conclusion is supported by analyses of tumour clonality. Studies of X chromosome inactivation patterns suggest that both monoclonal and polyclonal patterns of inactivation exist (Rabkin et al, 1995; Delabesse et al, 1997; Rabkin et al, 1997). A study of size heterogeneity in KSHV terminal repeats in nodular lesions demonstrated monoclonal, oligoclonal and polyclonal patterns of infection, implying that KSHV infection preceded tumour expansion (Judde et al, 2000).

1.2.5.2 Primary effusion lymphoma

First recognised in AIDS patients before the discovery of KSHV, primary effusion lymphoma (PEL) is a rare malignant effusion of the peritoneal, pleural or cardiac cavities. Hence, PEL was originally termed body cavity-based lymphoma (BCBL) (Knowles et al, 1989). The lymphoma cells combine features of immunoblastic and anaplastic large cell lymphomas (Gaidano et al, 1996). They display a large cytoplasm, irregular and pleomorphic nuclei with prominent nucleoli and significant size heterogeneity (Schulz 2001). The cells are generally of B cell origin, although rare cases of KSHV-positive PEL expressing T cell markers have been described (Said et al, 1999). Immunoglobulin locus rearrangement and patterns of immunoglobulin light chain expression demonstrate a monoclonal origin in most cases (Knowles et al, 1989; Cesarman et al, 1995b). Although they express very few markers of B cell differentiation, some activation markers including CD30, CD38, CD71 and epithelial membrane antigen are often present. A recent study, using microarrays to group B cell tumours by comparing their expression profiles, found that PEL gene expression was most similar to that of plasma cell tumours (Jenner et

al, 2003). These data support previous observations that PEL cells frequently express CD138 (Gaidano et al, 1997), an adhesion molecule whose expression is largely restricted to pre-B cells and plasma cells.

The KSHV genome is present at 50-150 copies per cell in the nuclei of PEL (Cesarman et al, 1995b; Arvanitakis et al, 1996; Renne et al, 1996a; Gessain et al, 1997), and PEL is a distinct neoplasm that is strongly associated with KSHV infection (Pastore et al, 1995; Carbone et al, 1996; Karcher et al, 1997). Nonethesless, co-infection with EBV is found in most PEL cases (Cesarman et al, 1995a; Cesarman et al, 1995b) and PEL is rarely found in the absence of AIDS. Thus EBV infection and immunosuppression probably contribute to the pathogenesis of PEL. However, the expression of EBV latent genes such as EBNA 2, EBNA 3 and LMP1 is restricted in these cells (Horenstein et al, 1997; Szekely et al, 1998; Callahan et al, 1999), making it less likely that EBV is driving their proliferation. Furthermore, examples of PEL containing only KSHV have been reported, from which cell lines have been derived (Arvanitakis et al, 1996; Said et al, 1996; Boshoff et al, 1998; Carbone et al, 1998; Carbone et al, 2000). When injected into nude mice, such cells can induce PEL-like lymphomas (Said et al, 1996; Boshoff et al, 1998). PEL is unusual amongst B cell malignancies in the absence of an association with any consistent genetic lesion such as mutations of genes encoding c-myc, ras or p53 (Cesarman et al, 1995a; Karcher et al, 1997; Gaidano et al, 1999). The absence of a common mutation, and the discovery of PEL in the absence of EBV support the concept that KSHV is directly responsible for transformation in these lymphomas.

1.2.5.3 Multicentric Castleman disease (MCD)

KSHV is variably found in a rare angiolymphoproliferative disorder termed multicentric Castleman disease (MCD) (Soulier et al, 1995; Corbellino et al, 1996). KSHV is found in more than 90% of AIDS patients with MCD but only 40% of HIV-seronegative MCD patients (Grandadam et al, 1997). In affected lymph nodes, KSHV is found in cells termed plasmablasts belonging to the B cell lineage that localise to the mantle zone of B cell follicles (Dupin et al, 1999; Katano et al, 2000). Since the presence of plasmablasts in MCD is specifically associated with KSHV infection, a distinct plasmablastic variant of MCD is recognised (Dupin et al, 2000). In some

cases of plasmablastic MCD, lymphoma can develop. Unlike PEL cells, co-infection with EBV has not been detected in plasmablasts (Dupin et al, 2000; Du et al, 2001).

1.2.6 Patterns of gene expression in KSHV

A biphasic life cycle is a common feature of herpesviruses (Cohrs et al, 2001). For neurotropic α-herpesviruses such as herpes simplex virus-1 (HSV-1), the initial lytic and productive infection of epithelial cells is followed by a quiescent and non-productive latent infection of neuronal cells. The virus thus establishes life-long infection of the dorsal root ganglia from where it can be periodically reactivated. The prototype β-herpesvirus, human cytomegalovirus (HCMV), establishes latency in myeloid progenitor cells of the bone marrow, and can be reactivated with particularly serious consequences in AIDS and transplant patients. The lymphotropic γ-herpesviruses, like EBV and KSHV, also establish latency in B cells and certain epithelial cells. Distinct patterns of gene expression, consistent with latent and lytic phases of infection have been observed in both KS lesions (Staskus et al, 1997) and PEL cells (Zhong et al, 1996; Renne et al, 2001). Latency serves two important purposes, in allowing the virus to establish persistent infection and to avoid immune surveillance. Furthermore, since lytic replication inevitably kills the host cell, the genes expressed during the lytic phase cannot be directly involved in transformation of the infected cell. The genes expressed in latency are therefore predicted to play a major role in the tumourigenesis associated with KSHV infection (Chang et al, 1996a).

Various chemicals, including tetradecanoyl phorbal acetate (TPA) and *n*-butyrate, can induce viral lytic replication in PEL cell lines (Arvanitakis et al, 1996; Renne et al, 1996b), and these chemicals have been used to assign KSHV genes to lytic and latent phases. The first genome-wide analysis of KSHV gene expression was made using the BC-1 PEL cell line (EBV and KSHV infected) (Sarid et al, 1998). Using DNA probes across the viral genome, Northern analysis was made of cells during normal culture (i.e. latent infection) and following TPA treatment (Sarid et al, 1998). On this basis, the genes were divided into three classes:

Class I	Expressed in normal culture and unaffected by TPA treatment
Class II	Expressed in normal culture but upregulated by TPA treatment
Class III	Expressed only upon TPA treatment

This study was the first to recognise the Class I "latency associated cluster" of LANA, vcyclin and vFLIP (Sarid et al, 1998). The latent classification of this cluster has been confirmed by their expression in a range of KSHV-infected tissues (Davis et al, 1997; Dittmer et al, 1998; Low et al, 2001), and their functions are discussed in greater detail elsewhere (Section 1.2.7.1). The Class II genes included small polyadenylated RNAs and most of the pirated viral genes (viral cytokines and signal transduction genes) (Sarid et al, 1998). The Class III genes largely consisted of viral structural and replication-associated genes. The kaposin (K12) gene was also identified as Class III (Sarid et al, 1998). However, *in-situ* hybridisation (ISH) applied to spindle cell populations demonstrated expression of kaposin in >85% of spindle cells, and confirmed its classification as a latent gene (Section 1.2.7.4) (Staskus et al, 1997). Most recently, microarrays have been used to study the kinetics of gene expression during induction of lytic replication (Jenner et al, 2001; Paulose-Murphy et al, 2001). These studies have largely confirmed the original classifications, and have provided a powerful means to assign gene function based on their expression profile and an understanding of the stages of the herpesvirus lifecycle.

1.2.7. KSHV latent genes implicated in viral pathogenesis

Seven KSHV genes are expressed during latent infection. All have the ability to modulate growth of infected cells and may therefore have a role in viral transformation and pathogenesis (Table 1.2).

1.2.7.1 The latency associated cluster

The adjacent ORFs: 71, 72 and 73 encode vFLIP, vcyclin and the latent nuclear antigen (LANA) respectively. Transcribed as two differentially spliced and polycistronic mRNAs from the same locus, the expression of these genes is controlled by a single promoter (Cesarman et al, 1996; Dittmer et al, 1998; Bieleski et al, 2001; Grundhoff et al, 2001). Latent transcript 1 (LT1) encodes LANA, vcyclin and vFLIP and LT2 encodes vcyclin and vFLIP (Talbot et al, 1999; Grundhoff et al, 2001; Low et al, 2001; Renne et al, 2001). LANA is translated from LT1, while both vFLIP and vcyclin are translated from LT2 (Section 1.4.7) by means of an internal ribosome entry site (IRES) (Fig. 1.3) (Bieleski et al, 2001; Grundhoff et al, 2001; Low et al, 2001). The promoter region is bi-directional, regulating constitutive expression of LT1 and LT2 to the left and expression of the lytic genes, K14 and vGPCR, to the right (Dittmer et al, 1998; Sarid et al, 1999; Talbot et al, 1999; Jeong et al, 2001). The functions of LANA and vcyclin are described below, while the role of vFLIP is explained in greater detail in Section 1.4.

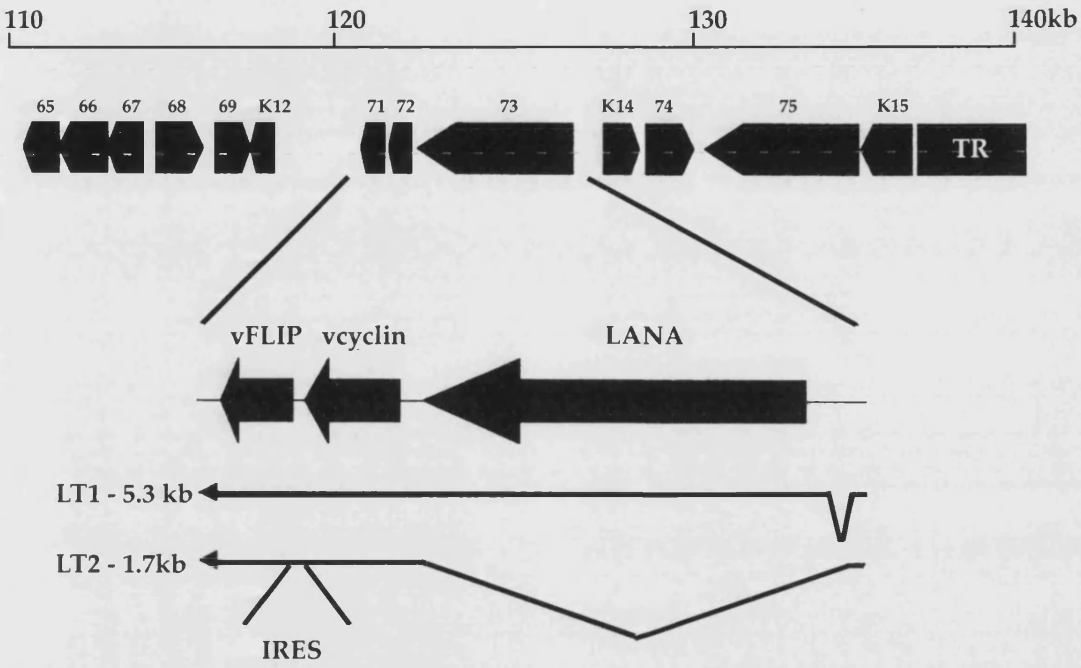


Figure 1.3. Transcription of the latent genes ORF 71, 72 and 73.

Map of the left end of the KSHV genome showing the alternative splicing of latent transcripts 71, 72 and 73 (taken from Bieleski et al, 2001). The coding direction of these genes is reversed because they are transcribed from the oppposite strand. Two spliced transcripts are observed in PEL cell lines: LT1 is tricistronic, containing vFLIP, vcyclin and LANA and LT2 is bicistronic encoding vFLIP and vcyclin. vFLIP is expressed from LT2 by virtue of an interal ribosome entry site (IRES).

1.2.7.2 Latency associated nuclear antigen (LANA)

LANA is a large multi-domain nuclear protein with diverse biological function. Immunoblot analysis using KS patient sera detected a LANA doublet of 222-224 kDa in latently infected KSHV-positive cell lines (Gao et al, 1996a; Gao et al, 1996b). The protein consists of 1,162 amino acids with three domains: (i) a proline rich N-terminal domain; (ii) an acidic internal repeat region; and (iii) the C-terminus which is responsible for the distinctive nuclear speckling pattern observed upon immunofluorescence assay (IFA) for LANA (Schwam et al, 2000). LANA interacts directly with host DNA and chromatin proteins to tether the viral episome to the host genome (Ballestas et al, 1999; Cotter et al, 1999; Szekely et al, 1999). During mitosis, this ensures efficient segregation of viral episomes to the daughter cells. LANA is therefore essential for the persistence of the viral episome during latency (Ballestas et al, 1999). LANA binds the viral episome at two 17 bp direct repeats found within the viral terminal repeats at the putative origin of replication (Ballestas et al, 2001; Garber et al, 2002; Lim et al, 2002). LANA also associates with Origin Recognition Complex (ORC) 1 and 2. These proteins are proposed to bind to the origins of DNA replication; the implication being that LANA has a role in viral replication.

In addition to these functions, LANA is capable of both activating and repressing transcription, through a variety of protein-protein and protein-DNA interactions (Renne et al, 2001; Jeong et al, 2001; Lim et al, 2002; An et al, 2002). LANA specifically activates the AP-1 response element to induce expression of cIL-6 (An et al, 2002; An et al, 2003a). These data are of particular significance in view of the importance of IL-6 in KSHV-associated disease (Sections 1.2.8.4; 1.4.7). LANA activates the HIV-1 LTR (Hyun et al, 2001) and its own promoter (Jeong et al, 2001). It can also enhance Sp-1-mediated activation of telomerase reverse transcriptase promoter elements (Knight et al, 2001). Since telomerase can stabilise telomere length, and its increased expression is associated with the immortalisation of cancer cells (Kim et al, 1994), these data imply a role for LANA in maintaining the proliferative potential of KSHV-infected cells. A LANA truncation mutant (803-990 amino acids) binds the hypophosphorylated form of retinoblastoma protein (pRb) in transfected cells to facilitate transactivation of promoters dependent on the pRb-E2F complex (Radkov et al, 2000). The pocket domain of pRb mediates this interaction,

and the data suggest that LANA competes with E2F for binding to this domain, with the consequence that E2F is released from pRb inhibition to activate genes involved in progression through the G1 cell cycle check-point. Such a mechanism may explain the cooperation between LANA and H-ras in transformation of primary rat embryo fibroblasts (Radkov et al, 2000). LANA also interacts with the p53 tumour suppressor to repress both its transcriptional and apoptotic activity (Friborg, Jr. et al, 1999). Since the p53 pathway can activate apoptosis in response to aberrant E2F activity, this interaction may also be required for transformation by LANA. In this respect, LANA function resembles that of viral oncogenes such as the simian virus 40 (SV40) large T antigen and E6/E7 of human papilloma virus (HPV).

The acetylation state of histone proteins can regulate the activity of adjacent promoters (Wolffe 1996; Giles 1998), since increased acetylation promotes an open chromatin structure allowing transcription factors and other components of the transcriptional machinery to access DNA regulatory elements (Korzus et al, 1998; Blobel 2000). Acetylation is carried out by histone acetylase (HAT) enzymes, and one of the best known activators of HATs is a protein called cAMP-response element-binding protein CREB)-binding protein (CBP). LANA binds CBP (Lim et al, 2001) to repress its transcriptional activity. LANA also interacts with RING3 and DEK1 (Platt et al, 1999), activating transcription factor (ATF)-4/CREB2 (Lim et al, 2000), and members of the mSin3 corepressor complex (Krithivas et al, 2000) and modulates the transcriptional activities mediated by these proteins. The interactions between LANA and chromatin remodelling proteins indicate a role for LANA in regulating global transcriptional activity of the infected cell.

Recently, an entirely novel function for LANA in deregulating β-catenin activity has been described (Fujimuro et al, 2003). When β-catenin accumulates, it is thought to enter the nucleus and activate transcription factors of the Lef and Tef family. Among target genes of Lef and Tef are Myc, Jun and cyclin D1 (He et al, 1998; Tetsu et al, 1999; Ben Ze'ev et al, 2000; Barker et al, 2000). Under normal circumstances, β-catenin is rapidly degraded, but Wnt can activate a signalling pathway to suppress proteasomal degradation of β-catenin. LANA binds and redistributes an upstream inhibitory component of this pathway, GSK-3β, leading to β-catenin accumulation.

Significantly, deletion of a small C-terminal region abrogated LANA binding to GSK-3β and prevented LANA stimulation of S-phase entry (Fujimuro et al, 2003). In the context of a variety of cancers that show aberrant β-catenin activity (Korinek et al, 1997), these data provide further evidence of transcriptional regulation by LANA that may be important in KSHV-associated pathogenesis.

1.2.7.3 viral Cyclin (vcyclin)

Like HVS, KSHV encodes a viral cyclin (vcyclin) that is homologous to cellular D-type cyclins, having 32% identity and 54% similarity to cyclin D2 (Russo et al, 1996; Chang et al, 1996b; Li et al, 1997). It shares a number of functional properties with its cellular counterparts: it binds and activates cdk6 and these complexes phosphorylate pRb, and promote cell cycle progression (Godden-Kent et al, 1997; Li et al, 1997). However, unlike the cellular D-type cyclins, the activity of vcyclin/cdk6 complexes cannot be limited by the CDK inhibitors $p16^{INKa}$, $p21^{CIP1}$ and $p27^{KIP1}$ (Swanton et al, 1997). The resistance of viral cyclins to $p27^{KIP1}$ inhibition has been explained by structural studies of murine herpesvirus 68 (MHV4)-encoded vcyclin (Card et al, 2000). These data demonstrate specific amino acid and conformational variations within viral cyclins that render the $p27^{KIP1}$ binding site non-functional (Card et al, 2000).

vcyclin/cdk6 complexes have a wider range of target substrates than cellular cyclin D2/cdk6. For example, vcyclin/cdk6 complexes phosphorylate substrates of cyclin E-cdk2 including histone H1, Id-2, and cdc25 (Ellis et al, 1999; Mann et al, 1999). vcyclin/cdk6 also phosphorylates the cdk2 substrate, $p27^{KIP1}$, resulting in its degradation by the 26S proteasome and relieving $p27^{KIP1}$-induced cell cycle arrest (Ellis et al, 1999; Mann et al, 1999). vcyclin/cdk6 also phosphorylates cellular Bcl-2, leading to apoptosis in cells expressing elevated levels of cdk6 (Ojala et al, 1999; Ojala et al, 2000). Interestingly, apoptosis was suppressed by co-expression of the viral lytic protein vBcl-2 (Section 1.2.8.4) but not cellular Bcl-2 (Ojala et al, 1999). vcyclin induces p53-dependent growth arrest in primary cells, but causes lymphomas in p53-null mice (Verschuren et al, 2002). Since LANA can disrupt p53 function and is expressed in conjunction with vcyclin, this may explain how the virus can benefit from a protein that is apparently pro-apoptotic.

Finally, vcyclin has been shown to mimic a function of cyclin A/cdk2 complexes and drive initiation of DNA replication *in vitro* (Laman et al, 2001). Using yeast-two-hybrid technology, this study identified ORC1 as an interacting partner of the vcyclin belonging to HVS. ORC1 interacts with a protein called cell division cycle (CDC) 6, which functions in pre-replication assembly and the initiation of DNA replication, events that are both regulated by cyclinA/cdk2 phosphorylation (Coverley et al, 2002). In complex with cdk6, vcyclin and HVS-cyclin phosphorylated both ORC1 and CDC6 *in vitro*, with the implication that vcyclin may stimulate the initiation of host genome replication (Laman et al, 2001). It is tempting to speculate that vcyclin also co-operates with LANA in ensuring replication of the latent viral genome (Hu et al, 2002).

1.2.7.4 Kaposin

Protein expression from the region encoding ORF K12 is complex and not completely understood (Sadler et al, 1999; Kliche et al, 2001). The T0.7 transcript is the most abundantly expressed transcript in KSHV latent infection, and was originally described as 0.7 kb, originating just upstream from K12 (Zhong et al, 1996). ISH analysis detected this transcript in the majority of KS spindle cells (Staskus et al, 1997), and in PEL cells (Sturzl et al, 1997). However, subsequent work detected larger K12 transcripts of 1.2-2.5 kb (Sadler et al, 1999). The larger transcripts originate upstream of the 0.7 kb transcript, and include two GC-rich direct repeat sequences called DR1 and DR2. The number of repeats is highly variable, explaining the heterogeneity of K12 transcripts (Sadler et al, 1999). These transcripts have Class III expression profile: being expressed in latency and upregulated upon TPA treatment (Sadler et al, 1999). Variant translational initiation from CUG and GUG codons within these transcripts gives rise to kaposin B and C *in vitro*, and although kaposin B was abundantly expressed in the PEL cell line BCBL-1 (Sadler et al, 1999), their function is not known. All transcripts contain the ORF K12 encoding the 60 amino acid hydrophobic membrane protein termed kaposin A (Russo et al, 1996; Sadler et al, 1999). Heterologous expression of kaposin A in rat-3 and NIH3T3 cells induces focus formation, and in NIH3T3 cells triggers anchorage independent growth and loss of contact inhibition (Muralidhar et al, 1998). Rat-3 cells expressing kaposin

A are tumourigenic in athymic nude mice, forming highly vascular sarcomas upon subcutaneous injection (Kliche et al, 2001). The transforming functions of kaposin A are mediated by a direct interaction with the guanine nucleotide exchange factor cytohesin-1 that regulates integrin activity (Kliche et al, 2001).

1.2.7.5 K15

Situated at the right end of the unique coding region, K15 is the positional homologue of the EBV gene, LMP 2A. The K15 gene consists of eight differentially spliced exons that encode a C-terminal cytoplasmic domain linked to a variable number of transmembrane domains (Glenn et al, 1999; Poole et al, 1999; Choi et al, 2000). Northern blot analysis showed weak K15 expression in PEL cells that was upregulated upon TPA treatment (Glenn et al, 1999; Choi et al, 2000). Latent protein expression has been confirmed in PEL cells and MCD (Sharp et al, 2002). The cytoplasmic domain of K15 contains a number of putative domains associated with signal transduction including: an SH2 domain, an SH3 domain and a TRAF-binding site (Glenn et al, 1999; Poole et al, 1999; Choi et al, 2000). In reporter assays, K15 strongly activated mitogen-activated protein kinase (MAPK) and weakly activated NF-κB pathways (Brinkmann et al, 2003). Yeast-two-hybrid analysis identified Hax-1 as an interacting partner of K15. This interaction was confirmed *in vivo*, and may play a role in inhibition of apoptosis (Sharp et al, 2002).

1.2.7.6 vIRF3

KSHV encodes four homologues of cellular interferon (IFN) regulatory factor (IRF) (K9/vIRF1, K11.1/vIRF2, K10.5/K10.6/LANA2/vIRF3, K10.1/vIRF4) (Section 1.2.8.6) (Jenner et al, 2002). ORF K10.5 encodes vIRF3, a protein that is latently expressed in KSHV-infected B cells, but not KS tissue (Rivas et al, 2001). It inhibits p53-dependent transactivation and apoptosis (Rivas et al, 2001), and also prevents apoptosis triggered by double-stranded RNA (dsRNA)-activated serine-threonine protein kinase (PKR) (see below) (Esteban et al, 2003). These data suggest a role for vIRF3 in protection of infected haematopoietic cells from immune surveillance in latency. The function of vIRF3 is probably related to deregulation of the immune system to promote survival of infected cells.

1.2.8 KSHV lytic genes implicated in viral pathogenesis

The latent genes of KSHV have important effects on growth-regulation and survival, suggesting a significant contribution to viral pathogenesis (Table 1.2). However, several lines of evidence also indicate a role for lytic reactivation in the development of KS. First, increased viral load is associated with progression to KS and is predictive of clinical outcome (Ambroziak et al, 1995; Whitby et al, 1995; Quinlivan et al, 2002). Second, treatment of AIDS-KS patients with ganciclovir, a drug that is active against KSHV lytic replication but not latent virus, reduces KS risk (Martin et al, 1999). Furthermore, regression of AIDS-KS due to highly active anti-retroviral therapy (HAART) is associated with reduced KSHV viral load (Sirianni et al, 1998; Wilkinson et al, 2002). Third, post-transplant KS usually results from reactivation of latent virus (Frances et al, 2000; Jenkins et al, 2002). Fourth, most KS spindle cells and MCD plasmablasts sustain latent KSHV infection, but in up to 20% the virus undergoes spontaneous reactivation (Zhong et al, 1996; Staskus et al, 1997; Staskus et al, 1999). Together, these data suggest that lytic reactivation is required to enhance the dissemination of virus and, as discussed below, may also modulate growth through paracrine mechanisms as a result of lytic gene expression.

1.2.8.1 K1

K1 is situated at the far left of the unique coding region. Its position is the equivalent of two herpesvirus oncogenes: STP of HVS (Jung et al, 1999) and LMP1 of EBV (Eliopoulos et al, 2001). Indeed, although K1 and STP do not share sequence homology, K1 substitutes for STP in immortalisation of common marmoset lymphocytes by a recombinant HVS (Lee et al, 1998). However, it should be noted that Tip, a related transforming gene belonging to HVS, was not removed from the recombinant virus. Nonetheless, K1 did independently induce focus formation in Rat-1 cells (Lee et al, 1998). Northern analyses report K1 transcripts expressed in PEL cell lines that are upregulated following TPA treatment (Lagunoff et al, 1999; Samaniego et al, 2001), and in KS tumour cells (Samaniego et al, 2001). A recent study using monoclonal antibodies raised against K1 confirmed early lytic expression in PEL cells and in MCD tissue, but K1 was not detected in KS samples (Lee et al,

2003). These data suggest there is an essential difference between K1 and STP/LMP1, because both are expressed in latency.

K1 is a transmembrane glycoprotein that resembles a single-domain Ig superfamily receptor. In fact, K1 binds to the heavy chains of the B cell receptor and prevents their expression on the plasma membrane (Lee et al, 2000). This function may indirectly prevent the display of KSHV viral antigens on B cell MHC class II, and may therefore represent an immune escape strategy of the virus. K1 also constitutively activates B cell signalling pathways via its C-terminal immunoreceptor tyrosine-based motif (ITAM) to stimulate nuclear factor of T cells (NFAT) (Lagunoff et al, 1999) and NF-κB activation (Samaniego et al, 2001; Prakash et al, 2002). Transfected K1 activates NF-κB in reporter based assays (Samaniego et al, 2001), while B lymphocytes from transgenic mice expressing K1 show increased NF-κB activity and the mice develop tumours that resemble spindle cell sarcomas (Prakash et al, 2002). Importantly, K1 contains two highly variable regions, which are used to classify the virus into four clades (A,B,C and D) (Hayward 1999; Zong et al, 1999; McGeoch 2001). Intriguingly, these regions were found to be targeted by CTL, and maybe subject to positive selection (Stebbing et al, 2003). From these data, it was inferred that K1 might act as an immune decoy, providing some evolutionary advantage to the virus.

1.2.8.2 Viral G-protein coupled receptor (vGPCR)

ORF 74 encodes a chemokine receptor homologue; viral G-protein coupled receptor (vGPCR) that has sequence similarity to the IL-8 receptor, CXCR2. It is expressed in the early lytic phase (Kirshner et al, 1999). Unlike its cellular counterparts, vGPCR signalling is constitutively active (Arvanitakis et al, 1997), but can be modulated by chemokine binding (Geras-Raaka et al, 1998; Gershengorn et al, 1998; Rosenkilde et al, 1999). vGPCR activates multiple signalling pathways, including the phosphoinositide-inositol triphosphate-protein kinase C (PKC) pathway leading to JNK/SAP activation (Arvanitakis et al, 1997; Sodhi et al, 2000), and the PI3-K/Akt pathway leading to NF-κB activation (Pati et al, 2001). Other groups have confirmed NF-κB activation by vGPCR in a variety of cell lines relevant to KSHV infection (Schwarz et al, 2001; Couty et al, 2001), leading to expression of NF-κB-dependent

genes including pro-angiogenic factors (VEGF), chemokines (IL-1β, IL-6, TNFα) and adhesion molecules (VCAM, ICAM-1, E-selectin) (Schwarz et al, 2001; Pati et al, 2001; Couty et al, 2001). vGPCR also activates NFAT (Pati et al, 2003), related adhesion focal tyrosine kinase (RAFTK) and lyn (Munshi et al, 1999).

Functional studies have revealed the extent to which vGPCR can modulate cell growth through activation of such signalling pathways. vGPCR transforms NIH3T3 cells (Bais et al, 1998), it enhances survival of primary endothelial cells (Couty et al, 2001; Montaner et al, 2001) and furthermore, vGPCR can immortalise human umbilical vein endothelial cells (HUVECs) (Bais et al, 2003). Transgenic mice expressing vGPCR develop multiple tumours that resemble KS lesions (Yang et al, 2000; Guo et al, 2003). Intriguingly, when the vGPCR transgene was controlled by a CD4 (primarily T cell) promoter, expression of vGPCR in the KS-like tumours was largely restricted to infiltrating T cells (Yang et al, 2000). These data support the concept that a paracrine component is important in the pathogenesis of KS tumours (Section 1.2.5.1). Significantly, the effects of vGPCR upon cell signalling pathways could be reconstituted in PEL cells and resulted in increased production of vIL-6 (see below) and VEGF (Cannon et al, 2003). It seems likely that vGPCR is an important component in KSHV-associated disease. In particular, it may act in a paracrine manner in concert with latent KSHV genes expressed in neighbouring cells (Montaner et al, 2003).

1.2.8.3 viral IL-6 (vIL-6)

cIL-6 functions as a growth factor for all KSHV-associated neoplasms (Miles et al, 1990; Screpanti et al, 1996; Asou et al, 1998), and high levels of cIL-6 have been observed in the tissues of patients with each of the KSHV-associated neoplasms (Ensoli et al, 1989; Leger-Ravet et al, 1991; Foussat et al, 1999). These observations suggest that cIL-6 is an important component of KSHV-associated pathogenesis. It is therefore interesting that the virus encodes a homologue of cIL-6. The viral IL-6 (vIL-6) is encoded by ORF K2 and has significant identity to cIL-6. The cIL-6 receptor consists of two subunits, gp130 and IL-6Rα, and cIL-6 has an absolute requirement for both subunits (Taga et al, 1997). However, vIL-6 requires only gp130 (Molden et al, 1997; Wan et al, 1999). This may allow for a broader spectrum of target cells since

IL-6Rα has a restricted expression profile and is downregulated by the IFN response, while gp130 is ubiquitously expressed (Taga et al, 1997). Structural studies have revealed essential differences in the way cIL-6 and vIL-6 interact with the receptor to explain these observations (Chow et al, 2001). vIL-6 stimulates growth in IL-6-dependent B cell lines (Moore et al, 1996a), and activates JAK/STAT and Ras-MAPK pathways (Molden et al, 1997; Osborne et al, 1999). vIL-6 is also an autocrine growth factor for PEL cells (Foussat et al, 1999), that protects against the cytostatic effects of IFNα (Chatterjee et al, 2002) and stimulates production of VEGF (Liu et al, 2001). When injected into nude mice, NIH3T3 cells stably expressing vIL-6 induce tumours that are more-highly vascularised than control cell-induced tumours (Aoki et al, 1999). vIL-6 may therefore be an important gene in KSHV-associated pathogenesis.

1.2.8.4 Viral Bcl-2 (vBcl-2)

ORF16 encodes a viral homologue of human Bcl-2, vBcl-2. (Sarid et al, 1997). The Bcl-2 family regulate apoptosis and thus play an important role in tissue homeostasis, embryogenesis and the immune respone (Chao et al, 1998). Cellular Bcl-2 prevents apoptosis through heterodimerisation with pro-apoptotic family members such as Bad, Bak and Bax in order to maintain mitochondrial integrity (Section 1.4.2). vBcl-2 is also anti-apoptotic, inhibiting apoptosis induced by vcyclin (Section 1.2.7.3) (Ojala et al, 1999), but interactions between vBcl-2 and cellular Bcl-2 family members have been difficult to prove (Cheng et al, 1997). One possible explanation for the anti-apoptotic function of Bcl-2 is suggested by the interaction between vBcl-2 and Diva, a pro-apoptotic protein that binds the caspase-9 regulator Apaf-1. vBcl-2 was found to antagonise the pro-apoptotic function of Diva (Inohara et al, 1998). Studies in adenovirus and baculovirus have helped to explain why viruses might employ strategies to prevent or delay apoptosis. These data demonstrated that virus-induced apoptosis was accompanied by a marked decrease in virus titre (Pilder et al, 1984; Clem et al, 1991; Clem et al, 1993). The inference being that, by delaying apoptosis, a virus might prevent premature lysis that would normally abort production of progeny virus. Furthermore, anti-apoptotic genes delay phagocytosis of the infected cell by antigen presenting cells (Koyama et al, 2000), and impede the immune response, thus maximising the window for viral replication.

1.2.8.5 Viral inhibitor of apoptosis protein (vIAP)

ORF K7 encodes viral inhibitor of apoptosis protein (vIAP) that is a homologue of the human survivin protein, survivinΔ-Ex3 (Wang et al, 2002). vIAP localises to the mictochondria (Wang et al, 2002), where it inhibits apoptosis through multiple stimuli including Fas, TRAIL, Bax, TNFα plus cyclohexamide, staurosporine and cermide (Wang et al, 2002; Feng et al, 2002). Mechanistically, vIAP bridges an interaction between cellular Bcl-2 and caspase-3 to inhibit caspase-3 activation (Wang et al, 2002). It therefore serves as an anti-apoptotic adaptor protein. vIAP also appears to control cytosolic Ca^{2+} concentration through a direct interaction with calcium-modulating cyclophilin ligand (CAML). By increasing cytosolic Ca^{2+}, vIAP was found to protect cells from mitochondrial damage and apoptosis (Feng et al, 2002).

1.2.8.6 vIRF1, vIRF2 and vIRF4

KSHV encodes three lytically expressed vIRFs. The IRFs are a family of transcription factors that regulate INF signal transduction through binding to interferon-stimulated response elements in the promoter of interferon-responsive genes. The IFNs represent an important part of the innate immune system antiviral strategy, stimulating the expression of a number of antiviral genes. Among these genes is PKR, a key mediator of antiviral and antiproliferative effects (Clemens et al, 1997). ORF K11.5 encodes viral-IRF2 (Burysek et al, 1999b), and vIRF2 can physically interact with PKR to block the antiviral effects of IFN (Burysek et al, 2001). vIRF2 also binds to the κB-consensus sequence and inhibits NF-κB activation, and interacts with RelA and p300 (Burysek et al, 1999b). K9 encodes vIRF1, which directly interacts with cellular IRF1 and IRF3 (Burysek et al, 1999a), p300 (Burysek et al, 1999a; Li et al, 2000), CRB (Seo et al, 2000) and p53 (Nakamura et al, 2001). Through effects on transcription mediated via these interactions, vIRF1 is able to transform NIH3T3 and Rat-1 cells (Gao et al, 1997; Li et al, 1998). vIRF1 is also a powerful antagonist of cellular IFN-mediated gene expression and antiviral effect (Zimring et al, 1998), and may also be important in regulating KSHV gene expression. Finally, vIRF4 is encoded within K10.1 and also subverts IFN signalling (Jenner et al, 2002).

ORF	Gene product	Function	Cell transformation	NF-κB activation	Expression pattern
K1		Down-regulates BCR and constitutively activates B cell signalling pathways.	Transformation in HVS. Focus formation in Rat-1 cells. Tumours in transgenic mice.	Demonstrated via reporter based assays and increased expression in transgenic mice.	Lytic
ORF 4	Viral complement binding protein (vCBP)	Inhibits the complement component of innate immune response (Spiller et al., 2003).	-	-	Lytic
K2	vIL-6	Growth factor. Activates gp130 independently of IL-6R.	-	-	Lytic
K3 and K5	Modulator of Immune recognition (MIR) 1 and MIR 2	Down-regulate immunoregulatory molecules such as MHC I (Ishido et al., 2000).	-	-	Lytic
K4, K4.1 and K6	Viral macrophage inflammatory proteins (vMIPs)	Homologues of human MIP1α, inducing angiogenesis and binding both CC and CXC receptors (Choi et al., 2001).	-	-	Lytic
K7	vIAP	Inhibits apoptosis.	-	-	Lytic
ORF16	vBcl-2	Inhibits apoptosis.	-	-	Lytic
K9	vIRF1	Deregulates IRF-mediated transcription. Binds p53 and CBP.	Transforms NIH3T3 and Rat-1 cells.	-	Lytic
K10.1	vIRF4	Deregulates IRF-mediated transcription.	-	-	Lytic
K10.5/K10.6 /LANA2	vIRF3	Deregulates IRF-mediated transcription.	-	-	Latent
K11.1	vIRF2	Deregulates IRF-mediated transcription.	-	Inhibits NF-κB binding to κB sequence	Lytic
K12	Kaposin A	Binds cytohesin-1.	Transforms Rat-3 and NIH3T3 cells.	-	Latent

Continued

ORF 71	vFLIP	Inhibits apoptosis. Binds IKKγ.	Transforms Rat-1 and NIH3T3 cells.	Binds IKKγ to persistently activate IKK. Demonstrated via reporter assays, kinase assays and EMSAs.	Latent
ORF 72	vcyclin	Constitutively active cyclin D homologue. DNA replication.	Induces lymphomas in p53-null mice.	-	Latent
ORF 73	LANA	Tethers viral episome. Antagonises p53 and pRb. Regulates transcription.	Transforms primary rats embryofibroblasts in conjunction with Ras.	-	Latent
ORF 74	vGPCR	Constitutively activates multiple transcription factors including JNK/SAP, NFAT and NF-κB.	Immortalisation of HUVECs. Tumours in transgenic mice.	Activates via PI3-K/Akt pathway. Up-regulates expression of NF-κB-dependent genes.	Lytic
K14	Viral OX-2 (vOx-2)	Activates production of inflammatory cytokines (IL-1β, TNFα, IL-6) (Chung et al., 2002).	-	-	Lytic
K15		Inhibits BCR signalling. Binds TRAFs and HAX-1.	-	Activation demonstrated reporter based assays.	Latent

Table 1.2. KSHV genes involved in viral pathogenesis, highlighting those capable of NF-κB activation and cell transformation.

Table is based on experimental data referenced in the text if not in the table. Adapted from Choi et al, 2001.

1.3 Regulation of NF-κB transcription factors

1.3.1 Rel and IκB protein families

Nuclear factor of κB (NF-κB) is the collective term for a group of dimeric transcription factors that regulate the expression of a wide range of genes involved in development, innate and adaptive immunity, and cellular survival. This set of genes includes an array of cytokines and adhesion molecules. It also includes molecules involved in the adaptive immune response and in migration, differentiation and maturation of lymphocytes. Finally, it contains genes that regulate cell growth and apoptosis (for review see (Pahl 1999) and (Ghosh et al, 2002)).

NF-κB was first discovered in 1986, as a protein binding the enhancer element within the kappa immunoglobulin (Ig) light-chain gene in B cells (Sen et al, 1986b). Soon afterwards, the same authors demonstrated NF-κB as an inducible transcription factor in a range of other cells (Sen et al, 1986a). NF-κB dimers are composed of different combinations of the five mammalian reticuloendotheliosis (Rel) family proteins. The Rel family (Fig. 1.4) share a conserved Rel homology domain (RHD) of 300 amino acids, which resembles two Ig domains (May et al, 1997). The RHD mediates the DNA binding and dimerisation of NF-κB subunits, and is also the binding domain for a family of inhibitory proteins, termed IκB (Ghosh et al, 1998). The IκB family (Fig.1.4) share a domain containing six or seven ankyrin repeats, through which they bind the RHDs (Whiteside et al, 1997). Two classes of Rel proteins are recognised. Class I members (RelA/p65, RelB and c-Rel) are synthesised as mature molecules, and class II members (p105/NF-κB1 and p100/NF-κB2) are synthesised as large precursor proteins containing an N-terminal RHD and a C-terminal ankyrin repeat domain. Prior to processing, p105 and p100 function as IκB-like molecules (Rice et al, 1992; Mercurio et al, 1993; Dobrzanski et al, 1995; Solan et al, 2002). Proteolysis cleaves their C-terminus to produce mature NF-κB subunits (p50 and p52 respectively) (Whiteside et al, 1997).

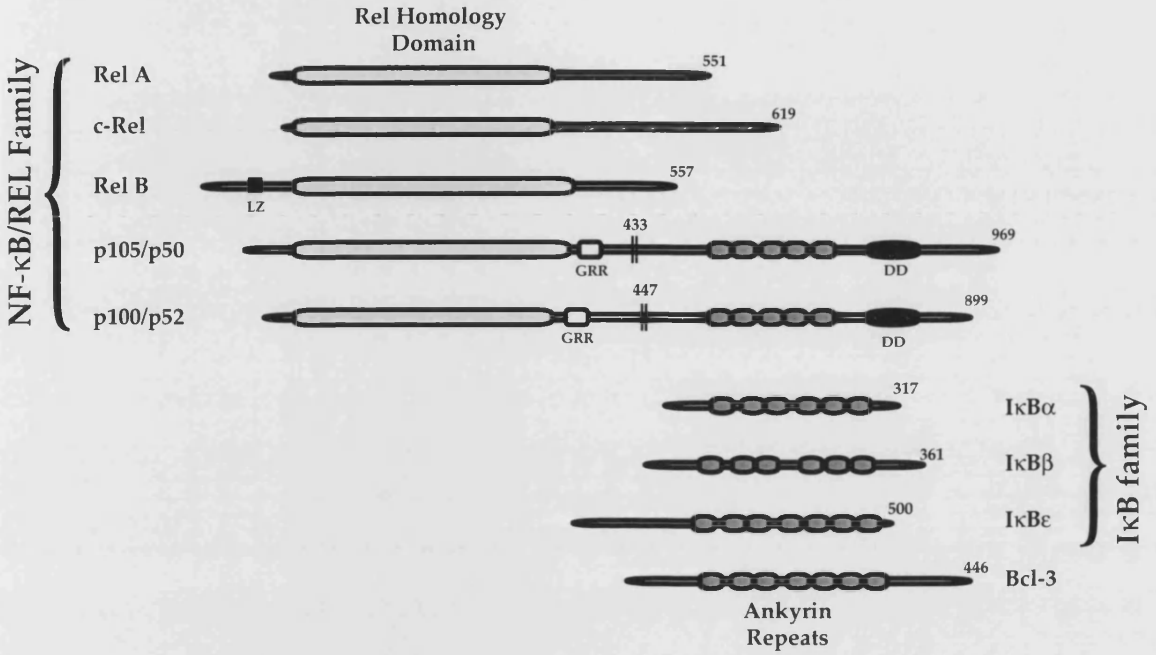


Figure 1.4. Schematic representation of the NF-κB/REL and IκB protein families.

The number of amino acids in each protein is listed on the right. The double lines in p105 and p100 indicate the C-terminus, following processing, of p50 and p52 respectively; LZ, leucine zipper; GRR, glycine rich region; DD, death domain. (adapted from Karin and Ben-Neriah, 2000)

1.3.2 NF-κB dimers

NF-κB is now known to exist in most cell types, binding to the common consensus sequence 5'-GGGRNNYYCC-3' (where R is purine and Y is pyrimidine) (Kunsch et al, 1992; Parry et al, 1994), that may regulate more than 150 genes (Pahl 1999). The RelA/p50 heterodimer is the most abundant NF-κB complex, but most combinations of NF-κB homo- and heterodimers have been described *in vivo* (Molitor et al, 1990; Kang et al, 1992; Ganchi et al, 1993; Parry et al, 1994; Hansen et al, 1994a). The one exception being RelB, which only forms heterdimers with p50 and p52 (Ryseck et al, 1992; Ryseck et al, 1995). Most dimers are transcriptionally active, however p50 and p52 lack the variable C-terminal transactivation domain that is present in RelA, RelB and c-Rel (Ghosh et al, 1998). Some data suggest that p50 and p52 homodimers are transcriptionally repressive (Kang et al, 1992; Plaksin et al, 1993; Brown et al, 1994; Hansen et al, 1994a; Hansen et al, 1994b), and this may be through passive occupancy of κB sites, competition with other NF-κB complexes (May et al, 1997) or through the recruitment of specific corepressor complexes (Zhong et al, 2002). However, p50 and p52 homodimers also bind specifically to the IκB-like proto-oncogene, Bcl-3, to form transcriptionally active complexes (Fujita et al, 1993; Bours et al, 1993). Interestingly, p52:Bcl-3 complexes bind the cyclin D1 promoter and stimulate cyclin D1 expression to potentiate G1 transition in human epithelial breast cells (Westerheide et al, 2001).

The various NF-κB dimers have different affinity for κB binding sites, allowing for the differential regulation of a wide range of genes (May et al, 1997). The first NF-κB:DNA crystal structure was solved for the p50 homodimer (Ghosh et al, 1995; Muller et al, 1995), demonstrating the DNA trapped between the folds of the RHD domains, which have been likened to butterfly wings. Contact with DNA is made by 10 loops at the tips of β-strands, mediating a strong but flexible interaction (May et al, 1997). Once bound to the promoter, NF-κB initiates transcription through the assembly of larger nucleoprotein complexes, termed enhanceosomes (Thanos et al, 1995). Studies on the IFN-β promoters have advanced understanding of this multistep process, which involves the assembly of DNA remodelling enzymes, sequence

specific transcription factors and co-activator proteins. The enhancer element of the IFN-β gene contains three domains, termed positive regulatory domains (PRDs) (Thanos et al, 1993). This enhancer is bound by three transcription factors, NF-κB, ATF-2/c-jun and IRF proteins that recognise PRDII, PRDIV and PRDIII-1, respectively. The DNA-remodelling protein HMG I(Y) binds the central region of PRDII (Thanos et al, 1992), and bends the DNA to allow binding of NF-κB and ATF-2/c-Jun (Yie et al, 1999). Further protein-protein interactions recruit IRF, general transcription factors and the co-activator p300/CBP into the complex (Munshi et al, 1998). Co-activators are non-DNA binding proteins that couple transcription factors to the basal transcription machinery and induce chromatin remodelling (Blobel 2000) (Section 1.2.7.2). The process of chromatin remodelling is the first step in promoter activation. It is therefore significant that RelA directly recruits p300/CBP through an interaction with its C-terminal transactivation domain (Perkins et al, 1997; Wadgaonkar et al, 1999).

Two distinct pathways regulate the transcriptional activity of NF-κB. The first applies to dimers composed of RelA, c-Rel and p50, and is termed the classical or canonical pathway. The archetype for this pathway was proposed following the discovery that latent NF-κB DNA-binding activity could be activated by treating cells with deoxycholate (Baeuerle et al, 1988). This led to the isolation of the inhibitory proteins, IκBα and β, that bind NF-κB and are thought to mask nuclear localisation signals (NLS) within the RHD (Whiteside et al, 1997). The simplest model for the classical pathway is that NF-κB is released when upstream signals induce proteolytic degradation of these inhibitory proteins (Henkel et al, 1993). NF-κB dimers then migrate to the nucleus and activate transcription. The second, or alternative pathway, controls the processing of p100. RelB is preferentially sequestered by full-length p100 (Dobrzanski et al, 1995; Solan et al, 2002), and specific stimuli can activate partial processing of p100 to release RelB heterodimers, and also generate p52. The regulation of these two pathways is the subject of the remainder of this section.

1.3.3 Classical NF-κB pathway

The classical pathway (Fig. 1.5), leading to the degradation of the small IκBs (IκBα, IκBβ and IκBε) can be activated by a variety of proinflammatory stimuli including bacterial LPS, negative strand viruses, ds-RNA, immunostimulatory sequences (ISS) of DNA, TNFα, IL-1 and antigens (Ghosh et al, 2002). It should be noted that, in some cases, both classical and alternative pathways are activated by the same stimulus (Section 1.3.4.3). Stimuli of the classical pathway trigger the activation of a specific IκB kinase (IKK) complex, which phosphorylates IκB proteins at two N-terminal residues (Ser32 and 36 for IκBα and Ser19 and Ser23 for IκBβ) (DiDonato et al, 1997; Mercurio et al, 1997; Regnier et al, 1997; Woronicz et al, 1997; Zandi et al, 1997). Importantly, a mutant of IκBα containing serine to alanine substitutions at positions 32 and 36, functions as a potent dominant negative inhibitor of the classical pathway, referred to as the super-repressor (Roff et al, 1996). Phosphorylated IκB is recognised by β-TrCP receptor protein and targeted for poly-ubiquitination at two major N-terminal arginine acceptor sites, by an E3 ubiquitin ligase enzyme (Alkalay et al, 1995b; Yaron et al, 1997). Phosphorylated and ubiquitinated IκB is thus targeted for degradation by the 26S proteosome (Brown et al, 1995; Chen et al, 1995). This process is rapid, such that all IκBα can be degraded within a few minutes (DiDonato et al, 1995; Alkalay et al, 1995a). The classical pathway therefore ensures a rapid response that is crucial for effective inflammatory and immunoregulatory processes.

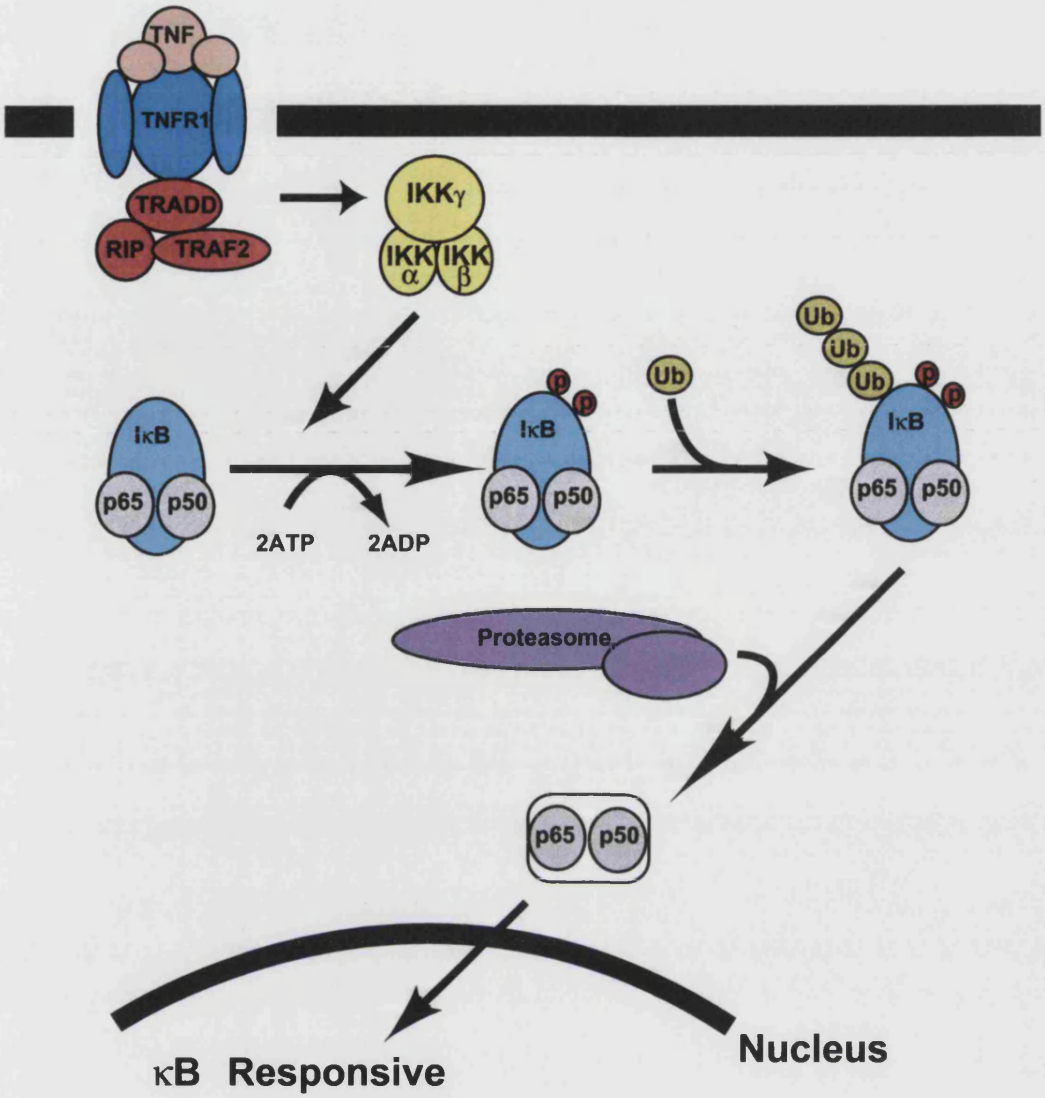


Figure 1.5. A model for the classical pathway of NF-κB activation.

The classical pathway involves activation of the IKK complex via membrane-bound receptors of the TNF-R superfamily. Phosphorylation of short IκB molecules by IKK targets these inhibitor molecules for ubiquination by the SCF ubiquitin ligase complex and processing by the 26S proteasome. NF-κB dimers are released and translocate to the nucleus to regulate transcription of κB dependent genes.

In addition to the IKK complex, several other modes of regulation exist in the classical pathway. For example, *de novo* IκBα was found to enter the nucleus and export NF-κB dimers (Brown et al, 1993; Sun et al, 1993; Chiao et al, 1994; Arenzana-Seisdedos et al, 1995), thereby limiting the NF-κB response. Another mechanism of regulation lies in the phosphorylation of NF-κB subunits to modify their transcriptional activity. Such a mechanism of control was first demonstrated for RelA following cellular stimulation (Naumann et al, 1994; Neumann et al, 1995), and subsequently shown for RelB and c-Rel. Such cytokine-inducible phosphorylation may regulate NF-κB recruitment of the enhanceosome (Ghosh et al, 2002). Further fine-tuning of the transcriptional activity of NF-κB may occur through ubiquitination, acetylation and the addition of the small ubiquitin-like modifier (SUMO) to key components within the NF-κB pathway (Freiman et al, 2003).

1.3.3.2 The IκB Kinase (IKK) complex

All known proinflammatory stimuli converge on the IKK complex (Ghosh et al, 2002). IKK is highly regulated, and in many respects holds the key to regulation of the entire NF-κB pathway. Discovery of IKK was therefore a major break-through in understanding NF-κB signalling. This discovery was driven by the knowledge that IκB degradation required specific stimulus-dependent phosphorylation. Using chromatography, Didonato et al, purified a 900 kDa protein kinase complex from the extracts of TNF-treated HeLa cells (DiDonato et al, 1997). This complex had all the hallmarks of a physiological IκB kinase: it was specific for IκBα; was able to discriminate against a mutant with threonine substitutions; and was rapidly activated by proinflammatory cytokines (DiDonato et al, 1997). Two polypeptides that coeluted with this IKK activity were identified by microsequencing, and cDNA cloning, as two closely related protein kinases, IKKα (IKK1) and IKKβ (IKK2) (DiDonato et al, 1997; Zandi et al, 1997). Concurrent experiments by Mercurio et al, yielded identical results (Mercurio et al, 1997). IKKα had previously been identified as a putative serine threonine kinase, termed conserved helix-loop-helix ubiquitous kinase (CHUK) (Connelly et al, 1995). At the same time, Regnier et al, identified CHUK as an interacting partner of NF-κB inducing kinase (NIK), with specific kinase activity towards IκB (Regnier et al, 1997). A third component, termed IKKγ, was discovered

soon afterwards by further purification and sequencing of IKK (Rothwarf et al, 1998). The mouse homologue of IKKγ was identified by complementation cloning, as a factor, termed NF-κB essential factor (NEMO), that could restore NF-κB activation in two cell lines defective in this activity (Yamaoka et al, 1998). IKKγ is also known as IKK-associated protein (IKKAP1) (Mercurio et al, 1999) and 14.7 interacting protein (FIP-3) (Li et al, 1999c).

IKKα and IKKβ are the catalytic components of the IKK complex. They are highly homologous proteins of 85 and 87 kDa respectively, sharing 50% identity and more than 70% similarity. IKKγ is non-catalytic, but essential for the assembly and activation of the high molecular weight IKK complex (Rothwarf et al, 1998; Yamaoka et al, 1998; Rudolph et al, 2000; Li et al, 2001). The N-terminus of IKKγ contains the binding site for the IKKα/β catalytic subunits, and this is probably within the first 100 amino acids although conflicting results have been published as to the precise location (Rothwarf et al, 1998; Mercurio et al, 1999; May et al, 2000; Poyet et al, 2000; Ye et al, 2000). IKKα and IKKβ dimerize via C-terminal leucine zipper motifs (Mercurio et al, 1997; Woronicz et al, 1997; Zandi et al, 1997), and associate with IKKγ via a motif in their extreme C-terminus (May et al, 2000; Hu et al, 2001). Both interactions are essential for IKK activity. The C-terminus of IKKγ is specifically required for IKK activation via pro-inflammatory stimuli (Rothwarf et al, 1998; Yamaoka et al, 1998; Makris et al, 2002; Ghosh et al, 2002). These data suggest that IKKγ may bridge interactions between IKK and upstream activators, since truncations and mutations within the C-terminus do not affect IKK complex assembly but do prevent its activation (Rothwarf et al, 1999). However, the precise role of IKKγ in activation of IKK has not yet been fully explained. More recently, the chaperone protein Hsp90 and a cochaperone called Cdc37 have been identified as additional components of the IKK complex (Chen et al, 2002). Hsp90 is unusual amongst chaperone proteins in its substrate specificity for enzymes involved in signal transduction. Established substrates include steroid hormone receptors (Picard et al, 1990), and a variety of protein kinases (Fisher et al, 2000; Sato et al, 2000; Goes et al, 2001). These proteins depend on Hsp90 for their maturation and stability (Maloney et al, 2002). A role for Hsp90 in maintaining the IKK complex is therefore consistent with its known range of functions.

1.3.3.3 The functions of IKKα and IKKβ

Phosphorylation appears to control the activity of the IKK complex, since protein phosphatases 2a (Fu et al, 2003) and 2cβ (Prajapati et al, 2003) inactivated IKK *in vitro*, and treatment of cells with the phosphatase inhibitor, okadaic acid, resulted in complex activation (DiDonato et al, 1997). Consistent with these data, an activation loop in IKKα and IKKβ was phosphorylated at two specific serine residues in response to TNFα and IL-1 (Delhase et al, 1999). Serine to alanine substitution of these residues in the IKKβ subunit resulted in a dominant negative mutant, while mutation to glutamate produced a constitutively active molecule (Mercurio et al, 1997). However, the same substitutions in IKKα had no effect on activation of the complex by TNFα or IL-1, but did inhibit RANK-induced expression of cyclin D1 and proliferation in mammary epithelial cells (Cao et al, 2001). Genetic experiments have also highlighted essential differences between the two catalytic subunits. Disruption of IKKβ (Tanaka et al, 1999; Li et al, 1999b; Li et al, 1999d) and IKKγ (Makris et al, 2000; Rudolph et al, 2000; Schmidt-Supprian et al, 2000) loci in mice resulted in embryonic lethality with severe liver degeneration, which was highly analogous to the $p65^{-/-}$ phenotype (Beg et al, 1995). $IKK\beta^{-/-}$ and $IKK\gamma^{-/-}$ mice were refractory to NF-κB activation by proinflammatory stimuli. In contrast, $IKK\alpha^{-/-}$ mice died perinatally with severe morphogenetic defects in keratinocyte proliferation and differentiation, but IκB degradation by proinflammatory stimuli was virtually unaffected (Hu et al, 1999; Takeda et al, 1999; Li et al, 1999a). Despite normal IκB degradation and nuclear translocation of NF-κB, $IKK\alpha^{-/-}$ mice were deficient in inducing several NF-κB-dependent mRNAs in response to IL-1 and TNFα (Li et al, 1999a; Li et al, 2002). Recent work has shown the nuclear recruitment of IKKα, where it bound to the promoter regions of NF-κB responsive genes in conjunction with RelA and CBP, following cytokine treatment of cells (Yamamoto et al, 2003; Anest et al, 2003). IKKα promoted chromatin acetylation via specific phosphorylation of Histone 3 (Yamamoto et al, 2003; Anest et al, 2003). This function was antagonised by IKKγ, which was also found to shuttle between cytoplasm and nucleus and compete with RelA and IKKα for binding to CBP (Verma et al, 2003). IKKα would therefore seem to have an important downstream role in

augmenting NF-κB-dependent gene expression, while activation loop phosphorylation of IKKβ is required to liberate NF-κB dimers from IκB inhibition.

1.3.3.4 Activation and Regulation of IKK

Although it is clear that the classical pathway of NF-κB involves IKKβ phosphorylation and activation, it is not yet understood how this signal is transduced. The pathway is best characterised for TNFα, which recruited the IKK complex to the TNFR1 upon receptor binding (Devin et al, 2000; Zhang et al, 2000; Chen et al, 2002). Genetic experiments have identified critical molecules in this process, including TRAF2 (Kelliher et al, 1998; Tada et al, 2001), TRAF5 (Tada et al, 2001) and the protein kinase RIP1 (Devin et al, 2000). TRAF2 and TRAF5 are proposed to recruit IKK, while RIP1 induces its activation, although the kinase activity of RIP1 was dispensable (Devin et al, 2000). Although these data fail to explain exactly how the IKK complex is activated, it seems most likely that IKKβ is either phosphorylated by upstream kinases that may be recruited by the C-terminus of IKKγ, or that a transautophosphorylation mechanism is responsible for its activation (see below).

Key elements of another important pathway that is required for B cell and T cell receptor-mediated activation of IKK have recently been elucidated (Yu et al, 2003). Using germline inactivation in mice, two groups demonstrated that a protein called mucosa-associated lymphoid tissue (MALT1)/paracaspase (MPC) is an essential regulator of NF-κB in lymphoid cells (Ruland et al, 2003; Ruefli-Brasse et al, 2003). The T cell receptor (TCR) signalling pathway activates IKK via two caspase-recruitment domain (CARD)-containing adaptor proteins, termed CARMA1 and Bcl-10 (Thome et al, 2003). Since CARD-containing proteins are involved in apoptotic pathways (Section 1.4.6), these proteins were initially assumed to mediate apoptosis. However, Bcl-$10^{-/-}$ mice had normal apoptotic responses but were immunodeficient and resistant to antigen receptor-mediated NF-κB activation (Ruland et al, 2001). Subsequently, Carma1 was found to interact with Bcl-10 (Gaide et al, 2001; Bertin et al, 2001), and the Carma1-deficient (Hara et al, 2003) and MPC-deficient mice (Ruland et al, 2003; Ruefli-Brasse et al, 2003) were found to have a similar phenotype to Bcl-$10^{-/-}$ mice. MPC contains an N-terminal death domain (DD), two Ig-like

domains and a C-terminal caspase-like domain. Chromosomal translocation of MPC [t(11;18)(q21;q21)] (Wotherspoon et al, 2002), like Bcl-10 [t(1:14)(p22;q32)] (Willis et al, 1999; Zhang et al, 1999), is associated with MALT B cell lymophoma, suggesting the two molecules may be components of a common signalling pathway. The most recent data confirm this speculation, and suggest that MPC functions downstream of Bcl-10 (Ruland et al, 2003; Ruefli-Brasse et al, 2003), which in turn lies downstream of Carma1 (Thome et al, 2003). Importantly, inactivation of MPC did not affect the alternative pathway of NF-κB activation (Ruland et al, 2003), suggesting these signalling components may be specific to antigen receptor mediated activation of the classical pathway.

On the basis of overexpression studies, many candidate IKKβ kinases have been suggested (Ghosh et al, 2002), but only three have stood the test of genetic ablation or RNAi. MAP/ERK kinase kinase (MEKK) $3^{-/-}$ MEFs were defective in TNFα- and IL-1-mediated IKK activation, and MEKK3 was found to function downstream of RIP1 and TRAF2 (Yang et al, 2001). In contrast, zeta protein kinase C (ζPKC) was found to be important in TNFα-mediated IKK activation in mouse lung cells but not in MEFs (Leitges et al, 2001). The MAP3 kinase, transforming growth factor [TGF]-β-activated kinase (TAK)-1 was shown to be critical in IL-1- and TNFα-induced activation of NF-κB in HeLa cells using RNAi (Takaesu et al, 2003). These data support *in vitro* observations that TAK1 could activate IKK via a novel ubiquitination mechanism (Deng et al, 2000; Wang et al, 2001). An ubiquitin-conjugating complex containing Ubc13 and Uev1, termed TRAF6-regulated IKK activator (TRIKA) 1, was biochemically purified and found to ubiquinate TRAF6 on lysine 63, leading to its activation. TRAF6 was then able to activate a second complex, TRIKA2, containing TAK1, TAB1 and TAB2, which in turn activated IKK. Intriguingly, ubiquitination of IKKβ (Carter et al, 2003) and IKKγ (Tang et al, 2003) has recently been suggested to activate IKK, and a deubiquitinating (DUB) enzyme, termed CYLD, was shown to negatively regulate IKK (Brummelkamp et al, 2003; Trompouki et al, 2003; Kovalenko et al, 2003). However, it is not clear whether these observations are connected to the ubiquitin-related activity of TAK1. In summary, accumulating evidence suggests that several different mechanisms exist to activate IKK, depending on cell type and stimulus.

The second mechanism by which IKKβ may be activated is via induced proximity and transautophosphorylation. It has been shown that enforced oligomerisation of the N-terminus of IKKγ, or truncated IKKα and IKKβ mutants lacking their C-terminus, can activate IKK, and it is proposed that RIP1 may mediate the oligomeristation of IKK *in vivo* (Poyet et al, 2000; Inohara et al, 2000). Overexpression of active IKKβ leads to activation via autophosphorylation (Zandi et al, 1998), and it is suggested that the ability of IKKβ to oligomerise and transautophosphorylate is essential for IKK activation (Tang et al, 2003). It is also possible that the two mechanisms are not mutually exclusive. The most recent data suggest that IKKγ interacts with IKKα and IKKβ as a tetramer (Tegethoff et al, 2003), which hold the kinase subunits in position. It seems possible that post-translational modification (be it ubiquitination and/or phosphorylation) of IKK subunits might trigger a conformational change within the complex that facilitates IKKβ autophosphorylation. Crystal structure analysis of inactive and active IKK complexes may reveal answers to this question.

1.3.4 Alternative NF-κB pathway

The alternative pathway regulates the processing of p100 (Fig. 1.6). This discovery derived from the observation that germline inactivation of the nfkb2/p100 gene (Franzoso et al, 1998; Caamano et al, 1998) resulted in a phenotype that bore remarkable similarity to $NIK^{-/-}$ (Yin et al, 2001) and aly/aly (Koike et al, 1996) mice. These mice are characterised by the systemic absence of lymph nodes and Peyer's patches, disorganised splenic and thymic architectures, lack of germinal centres and defective B cell-mediated responses resulting in immunodeficiency. Since the alymphoplasia (aly) phenotype is attributed to a Gly855-to-Arg substitution in the C terminus of NIK (Shinkura et al, 1999), this suggested that NIK might have a role in p100 processing (Xiao et al, 2001b). Overexpression of NIK was subsequently shown to induce the processing of p100, a function which was inhibited by the aly mutation (Xiao et al, 2001b). Soon afterwards, Senftleben et al, demonstrated a role for IKKα in this pathway using $IKK\alpha^{-/-}$ haematopoietic stem cells to reconstitute lethally irradiated mice (Senftleben et al, 2001; Kaisho et al, 2001). These chimeras displayed similar defects in B cell maturation and lymphoid architecture to those of aly/aly, $NIK^{-/-}$ and $nf\kappa b2^{-/-}$ mice. Moreover, B cells derived from $IKK\alpha^{-/-}$ mice exhibited a specific deficiency in p100 processing that could not be rescued by the ectopic expression of NIK (Senftleben et al, 2001). *In vitro* kinase assays suggested that IKKα can phosphorylate p100 directly, leading to ubiquitin-dependent generation of p52 (Senftleben et al, 2001). These data implied the existence of a specific pathway, with IKKα lying downstream of NIK, which regulates p100 processing.

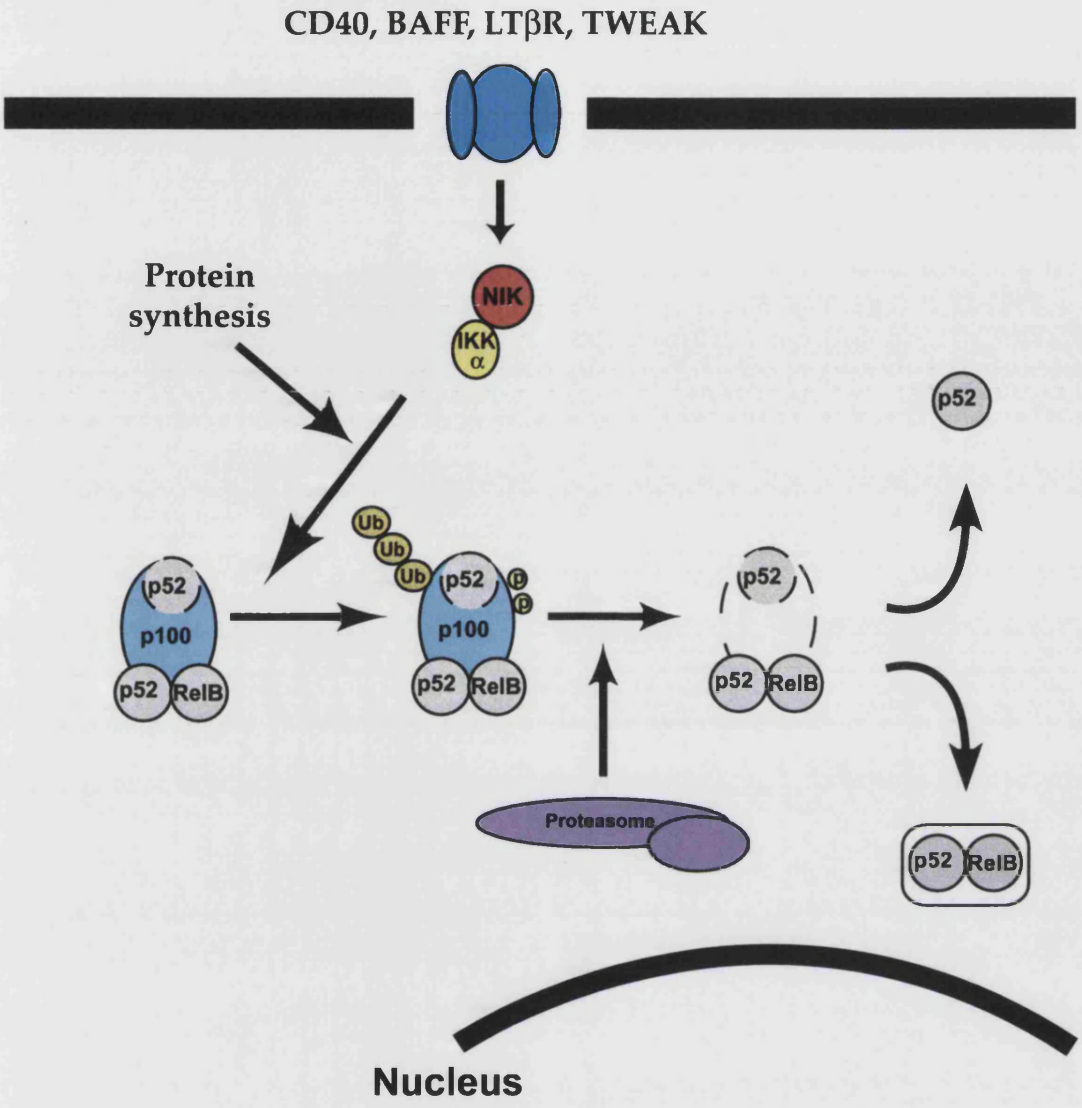


Figure 1.6. A model for the alternative pathway of NF-κB activation.

The alternative pathway involves activation of NIK and IKKα protein kinases via membrane-bound receptors of the TNF-R superfamily. Phosphorylation by IKKα marks p100 for ubiquination by the SCF ubiquitin ligase complex and partial processing by the 26S proteasome. NF-κB dimers containing RelB are released and p52 is generated from the processing, liberated dimers are thought to be free to translocate to the nucleus.

1.3.4.2 p100 processing

Unlike the constitutive processing of p105, the processing of p100 is tightly regulated (Betts et al, 1996; Heusch et al, 1999). The lack of constitutive p100 processing has been attributed to a processing inhibitory domain (PID) located within a death domain (DD) at the C-terminus, since p100 truncation mutants lacking the DD/PID are constitutively processed (Xiao et al, 2001b). It is therefore interesting that genetic alterations of the nfκb2 gene that are associated with lymphoid neoplasms have been shown to generate proteins that lack this region (Rayet et al, 1999). Processing of p100 is also regulated by a glycine-rich region (GRR) at amino acid 346-377 (Heusch et al, 1999). Translocation of this GRR alters the site of proteasomal processing (Heusch et al, 1999). Like the small IκB proteins, inducible phosphorylation of p100 upon two C-terminal serine residues (S866 and S870) leads to ubiquitination (Fong et al, 2002a; Fong et al, 2002b). The DD of p100 is required for the phosphorylation-induced recruitment of β-transducing repeat-containing protein (β-TrCP), a component of the SKP1-cullin-F box (SCF) ubiquitin ligase complex which catalyses p100 polyubiquitination (Fong et al, 2002a). Yeast two-hybrid analysis identified S9, a component of the 19S subcomplex of the 26S proteasome, as an interacting partner of the C-terminus of p100 (Fong et al, 2002b). In mammalian cells, this interaction was NIK-inducible and led to the partial processing of p100 to generate p52.

1.3.4.3 Activation of the alternative pathway

Four receptors belonging to the TNF receptor superfamily have been shown to induce p100 processing. B-cell activating factor (BAFF) is critical for the development and survival of peripheral B cells (Schiemann et al, 2001; Gross et al, 2001). BAFF receptor 3 (BR3) is the only specific receptor for BAFF and has been shown to induce p100 processing (Claudio et al, 2002; Kayagaki et al, 2002). Interestingly, transgene mediated overexpression of BAFF leads to B cell hyperplasia and a systemic lupus erythematous-like condition in mice (Mackay et al, 1999; Gross et al, 2000; Khare et al, 2000). Lymphotoxin-β receptor (LTβR) signalling also induced p100 processing (Saitoh et al, 2002; Dejardin et al, 2002; Muller et al, 2003; Mordmuller et al, 2003). LTβR is expressed on stromal cells, and a crucial role for this receptor in the development of lymphoid tissue has been suggested by genetic ablation in mice,

which fail to form lymph nodes and Peyer's patches (Shakhov et al, 2001). CD40 is expressed on B cells and triggers their clonal expansion and differentiation (Calderhead et al, 2000). CD40 ligation also induces p100 processing (Coope et al, 2002). Finally, TNF-like weak inducer of apoptosis (TWEAK), is expressed on human monocytes, and was also found to induce the alternative NF-κB pathway (Saitoh et al, 2003). These data suggest that the alternative pathway activates a transcriptional programme that is essential to B cell function. There were four essential similarities between the induction of p100 by these receptor:ligand interactions. First, genetic experiments have demonstrated that IKKγ and IKKβ were not required for p100 processing, indicating that this pathway was functional in the absence of a classical IKK complex. Second, p100 processing was translation-dependent, indicating a requirement for *de novo* protein synthesis. Third, in each case the kinase activities of IKKα and NIK were essential. Fourth, both classical and alternative pathways were activated by these stimuli (see below).

Using time course assays in MEFs, Muller et al, made a series of observations to highlight the essential differences between the classical and alternative pathways (Muller et al, 2003). In this study, TNFα treatment induced immediate degradation of IκBα that was considered to be indicative of classical pathway activation, but p100 and p52 levels were unaffected. In contrast, although LTβR agonistic antibody induced some early IκBα degradation, p100 was found to decrease after 4h and this was matched by an increase in p52. This late processing of p100 was considered to be indicative of alternative pathway activation. EMSA supershift and nuclear:cytoplasmic fractionation assays were used to analyse nuclear NF-κB dimers during the LTβR response. RelA-containing dimers dominated in the nucleus during the early time points, but were replaced by RelB-containing dimers at later time points. The authors used MEFs with single gene deletions to isolate each pathway. Thus, $IKK\gamma^{-/-}$ and $IKK\beta^{-/-}$ cells were permissive for p100 processing and RelB dimers were observed in the nucleus, but IκBα degradation and translocation of RelA at early time points did not occur in these cells. Using $IKK\alpha^{-/-}$ and *aly/aly* mice, signigicant levels of RelA translocation were found, but dimers containing RelB were not observed. These data correlate well with reports of distinct patterns of gene expression in $IKK\alpha^{-/-}$ and $IKK\beta^{-/-}$ mice (Senftleben et al, 2001; Dejardin et al, 2002).

Thus, expression of proinflammatory molecules (including macrophage inflammatory factor (MIP)-1β, MIP2 and VCAM-1) was dependent on IKKβ, while the expression of secondary lymphoid tissue chemokine (SLC), B lymphocyte chemoattractant (BLC), Ebl-1-ligand chemokine (ELC) and BAFF were dependent on IKKα (Dejardin et al, 2002). Together, these data suggest that the alternative and classical pathways can operate independently. Furthermore, the two are distinct in molecular mechanism, in time course and also in the specific NF-κB dimers and therefore genes that are activated. The data suggest that p100 processing activates RelB transcriptional activation, and this is consistent with the function of p100 as a specific inhibitor of RelB-containing dimers (Solan et al, 2002). Further analysis will be required to explain why this pathway is dependent on protein expression. It is possible that expression of an essential factor is necessary, or that processing is linked to the *de novo* expression of p100. Since p100 expression is activated by the classical pathway (Liptay et al, 1994; Sun et al, 1994), this may explain the delay in activating the alternative pathway.

1.3.4.4 Viral activation of the alternative pathway

Two viral oncoproteins have been shown to activate both classical and alternative NF-κB pathways: Tax of Human T cell leukaemia virus (HTLV)-1, and LMP1 of EBV. HTLV-1 is associated with an acute T-cell malignancy termed, adult T-cell leukaemia (ATL) (Poiesz et al, 1980; Yoshida et al, 1982), while EBV is the etiological agent for a number of neoplastic diseases including Burkitt's lymphoma, classical Hodgkin's lymphoma and nasopharyngeal carcinoma (Table 1.1). LMP1 is a membrane-associated protein with six hydrophobic transmembrane domains and a C-terminus of 200 amino acids (Hatzivassiliou et al, 2002). It is one of five latent genes essential for transformation of B cells by EBV (Kaye et al, 1995). LMP1 transformed Rat-1 fibroblasts (Wang et al, 1985), and induced lymphomas when expressed under control of Ig heavy chain and enhancer promoter in transgenic mice (Kulwichit et al, 1998). LMP1 is a functional homologue of CD40 (Zimber-Strobl et al, 1996; Kilger et al, 1998), but has ligand-independent constitutive activity (Gires et al, 1997). Via its C-terminus, LMP1 activates both NF-κB and AP-1 transcription factors, and this domain is required for transformation by LMP1 (Eliopoulos et al, 2001). The activation of NF-κB is proposed to be critical for the transforming activity of LMP1

(Izumi et al, 1997), and this conclusion is supported by the observation that the IκBα super-repressor prevented LMP1-mediated transformation of Rat-1 cells (He et al, 2000). Two sub-domains in the C-terminus of LMP1, termed carboxy-terminal activating regions (CTARs), are responsible for recruiting adaptor molecules and activating NF-κB (Huen et al, 1995; Mitchell et al, 1995; Devergne et al, 1996). Recent experiments using point mutants have isolated the function of each CTAR: CTAR1 was responsible for triggering IκBα phosphorylation, and therefore the classical pathway, and CTAR2 was required for induction of p100 processing and RelB:p52 nuclear translocation (Saito et al, 2003; Atkinson et al, 2003; Eliopoulos et al, 2003). In other respects, LMP1 activation of the alternative pathway resembled that of CD40 and the other TNFRs, including the requirement of NIK and IKKα, for protein synthesis, and for proteasomal activity.

Tax is likely to be the oncogenic component of HTLV-1, inducing transformation of $CD4^{+ve}$ cord blood cells *in vitro* (Grassmann et al, 1992) and soft tissue tumours in transgenic mice when expressed under control of the HTLV-1 LTR (Nerenberg et al, 1987). Tax also potently induced the aberrant expression of a large number of cellular genes (Ressler et al, 1996), many of which are regulated via NF-κB (Sun et al, 1999). Furthermore, Tax activated NF-κB through a direct interaction with IKKγ (Harhaj et al, 1999; Jin et al, 1999; Chu et al, 1999; Xiao et al, 2000) that induced persistent IKK activity (Geleziunas et al, 1998; Uhlik et al, 1998; Yin et al, 1998; Chu et al, 1998). Various lines of evidence suggest that this NF-κB activation is critical for transformation by Tax. First, a tax mutant defective in NF-κB activation was unable to immortalise primary human T cell *in vitro* (Robek et al, 1999). Second, overexpression of p100 together with Tax blocked the ability of Tax to transform Rat-1 cells (Yamaoka et al, 1996). Third, protection against growth-factor-withdrawal-mediated apoptosis by Tax correlated with NF-κB activity (Iwanaga et al, 1999; Tsukahara et al, 1999). Fourth, an inhibitor of IκB phosphorylation, termed Bay 11-7082, induced apoptosis in HTLV-1 infected cells (Mori et al, 2002). The fact that co-expression of p100 could block Tax-mediated transformation is particularly interesting, and suggests that the IκB function of p100 can block transformation. Subsequently, Tax has been demonstrated to induce p100 processing (Xiao et al, 2001a). However, Tax activation of the alternative pathway is unorthodox. In contrast

to other known activators of p100 processing, Tax functioned independently of NIK but required IKKγ. Tax bound directly to both p100 and IKKγ, seeming to bridge an interaction between the two proteins that served to recruit p100 to IKKα (which was essential for processing), which in turn induced phosphorylation-dependent ubiquitination and processing of p100.

1.4 KSHV-encoded vFLIP

1.4.1 Introduction

KSHV ORF71 encodes vFLIP. To explain the discovery and function of vFLIP it is helpful to understand the pathways involved in apoptosis.

1.4.2 Apoptosis and the caspase family

Apoptosis, or programmed cell death is an essential process for the survival of all multi-cellular organisms. Apoptotic death allows the removal of old, damaged or superfluous cells and is central to many important processes, including regulation of the immune system, embryonic development and metamorphosis. Apoptosis is a highly regulated process characterised by cytoskeleton disruption, shrinking of cells, condensation of nuclei and internucleosomal degradation of DNA (Kerr et al, 1972). The ordered series of biochemical events that culminate in apoptosis can be triggered either at the cell membrane (extrinsic pathway) or through various forms of intracellular stress (intrinsic pathway). In each case, large multi-protein complexes are formed and a family of proteins called caspases are activated (Bratton et al, 2000). The caspase family was discovered through homology to the pro-apoptotic gene, *ced-3*, encoded by the nematode worm, *Caenorhabditis elegans* (Yuan et al, 1993; Xue et al, 1996). The first to be identified was interleukin-1β converting enzyme (ICE) (Thornberry et al, 1992; Cerretti et al, 1992). The caspases are aspartate-specific cysteine proteases that are synthesised as zymogens. At least 14 members of the caspase family are now recognised and divided into three subgroups on the basis of their structure and function (Alnemri et al, 1996). Group I (caspase-1, -4, -5, -11, -12, -13, and -14) are ICE-related and involved in the inflammatory response. Group II (caspase-2, -3, and -7) and group III (caspase-6, -8, -9, and -10) are directly involved in apoptosis. Group III are known as 'initiator' caspases that are recruited to upstream signalling complexes by virtue of their long pro-domains. The group II, or 'effector' caspases, are the substrates for the initiator caspases. Once activated by cleavage,

these enzymes cleave structural and regulatory proteins within the cell and are responsible for many of the dramatic morphological features of apoptosis.

The group III caspases receive and amplify the apoptotic signal. Their activation therefore represents a crucial step in the commitment of a cell to apoptosis. The intrinsic pathway (Fig. 1.7) is activated to eliminate cells in response to ionising radiation, cytotoxic drugs, growth factor withdrawal and other forms of intracellular damage (Boatright et al, 2003). Such stimuli lead to the permeabilisation of the mitochondrial outer membrane and subsequent release of cytochrome c and other cytocidal proteins. Also released are Smac/Diablo and Omi/HtrA2, which prevent the IAPs (see below) from inhibiting capase activity. Important regulators of the intrinsic pathway are the Bcl-2 family members, which are broadly divided into two categories according to whether they promote or inhibit apoptosis (for review see (Adams 2003),(Kuwana et al, 2003) and (Newmeyer et al, 2003)). When released, cytochrome c forms a 700 kDa complex called the apoptosome (Acehan et al, 2002), containing apoptotic protease-activating factor 1 (Apaf-1), procaspase-9 and dATP/ATP. Caspase-9 is activated within the apoptosome and initiates the caspase cascade (Cain et al, 1999; Zou et al, 1999). By contrast, the extrinsic pathway (Fig. 1.7) is required for the removal of superfluous cells during development, immune system regulation and removal of tumour cells by cytotoxic T cells. It involves the activation of capase-8 via specialised membrane-associated receptors, belonging to a subset of the tumour necrosis factor receptor (TNFR) family termed death receptors (DRs). The DRs and extrinsic pathway are described in more detail below.

To prevent uncontrolled apoptosis resulting in tissue damage and disease, apoptosis is regulated by at least three groups of inhibitors. It is pertinent that KSHV encodes one member of each family. First, the inhibitor of apoptosis (IAP) family bind directly to caspases-3, -6, -7 and -9 to inhibit their function. KSHV encodes vIAP/K7 (Section 1.2.8.5). Second, the anti-apoptotic Bcl-2 family members regulate the intrinsic pathway of apoptosis. KSHV encodes vBcl-2 (Section 1.2.8.4). The third is the FLIP family, and vFLIP encoded by KSHV is focus of this section.

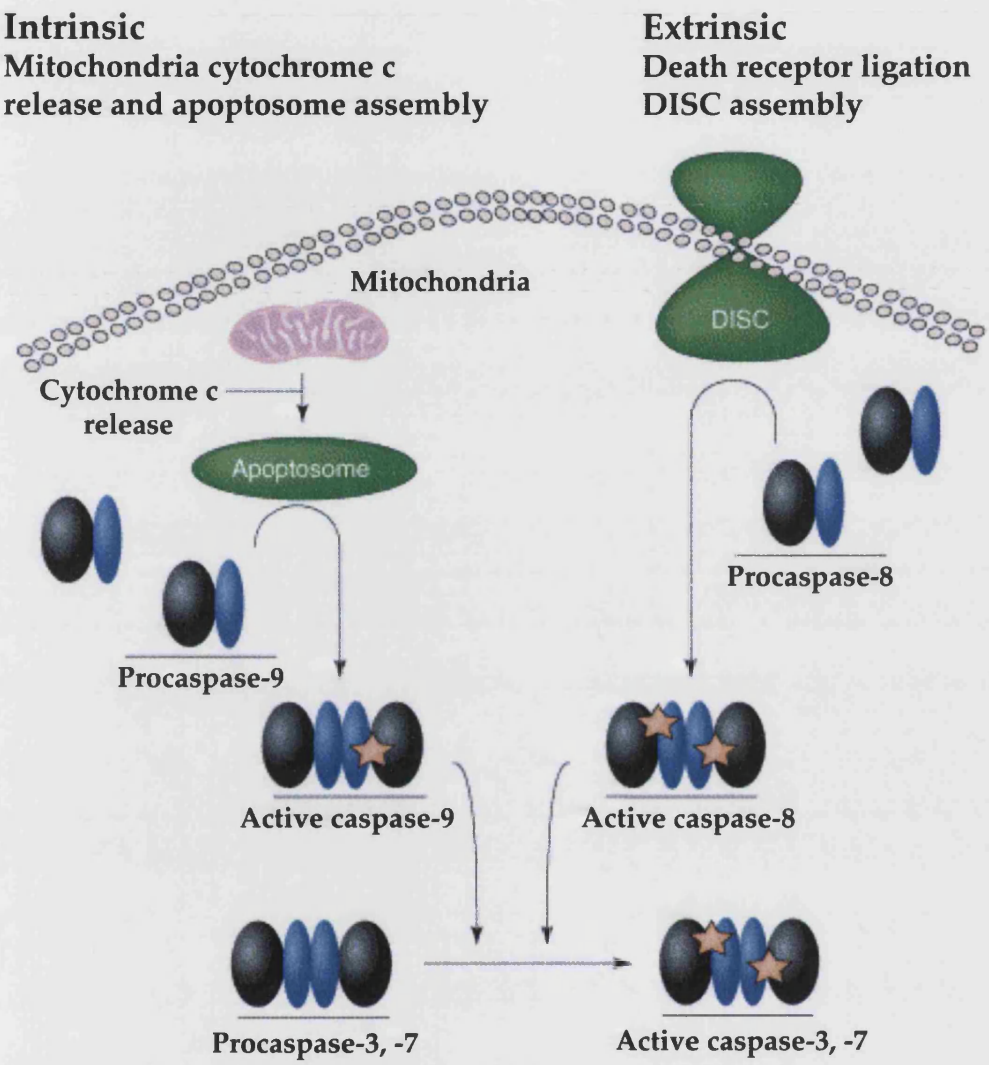


Figure 1.7. Overview of caspase activation by intrinsic and extrinsic pathways.

Engagement of either the extrinsic or the intrinsic death pathways leads to the activation of the initiator caspases by dimerization at multiprotein complexes. In the extrinsic pathway, the DISC is the site of activation for caspase-8 and caspase-10. The active sites are represented by orange stars. Stimulation of the intrinsic pathway leads to activation of caspase-9 at the apoptosome. Caspase-9 is shown as having one active site as seen in its crystal structure. However, the number of active sites *in vivo* is unknown. Following activation, the initiator caspases then cleave and activate the executioner caspases-3 and -7. (taken from Boatright and Salvesen, 2003)

1.4.3 Death receptor mediated apoptosis

The DR family includes TNFR1, CD95 (Fas/Apo-1), TNF-related apoptosis-mediating protein (TRAMP; also DR3), TNF-related apoptosis-inducing ligand receptor 1 (TRAIL-R1; DR4), TRAIL-R2 (DR5), DR6, nerve growth factor receptor (NGF-R) and ectodermal dysplasia receptor (EDA-R). These proteins are characterised by their N-terminal cysteine-rich domains (CDRs) that determine ligand specificity (Bodmer et al, 2002), and by a distinctive stretch of 60-70 amino acids in the C-terminus termed the death domain (DD) that is essential for the induction of apoptosis (Ashkenazi et al, 1998). Signalling through Fas has been particularly well characterised. Triggering of the receptor upon engagement of its ligand (CD95L) results in receptor trimerisation and DD-mediated recruitment of the adaptor molecular, FADD. In turn, FADD recruits caspase-8 or caspase-10 via a homophilic death effector domain (DED) interaction to form the so-called death-inducing signalling complex (DISC) (Scaffidi et al, 1999a). Within the DISC, induced proximity stimulates autoproteolytic cleavage of the initiator caspases to release the active subunits that cleave and activate the effector caspases. Cellular FLIP (cFLIP) is recruited to the DISC to inhibit the release of active caspase-8 subunits (Irmler et al, 1997) and thus blocks apoptosis (Section 1.4.5).

The molecular mechanisms controlling TNFR1-mediated apoptosis are more complex. In particular, confusion has arisen over the ability of TNFR1 to activate both apoptosis and proliferative/pro-survival pathways. However, a model has recently been proposed by Micheau and Tschopp, which may clarify some of the previous confusion (Micheau et al, 2003). Using time course and fractionation assays, they demonstrate the sequential formation of two distinct signalling complexes. Upon receptor engagement, complex I composed of TNFR1, TNFR-associated death domain (TRADD), receptor interacting protein (RIP), TNFR-associated factor (TRAF) 2, and c-IAP1 forms at the membrane and transduces a signal activating NF-κB. Complex I formation appears to be transient, for within an hour TRADD, RIP and TRAF2 dissociate from TNFR1 and the receptor undergoes endocytosis (Jones et al, 1999). Complex II (the DISC), consisting of TRADD, FADD, caspase-8 and variable levels of $cFLIP_L$ (Section 1.4.5) and cIAP1 subsequently forms in the cytosol. The

authors argue that the levels of cIAP1, and in particular $cFLIP_L$, dictate whether this DISC is able to induce apoptosis or not (Micheau et al, 2003). Since the expression of both anti-apoptotic proteins is regulated by NF-κB (Wang et al, 1998; Micheau et al, 2001; Kreuz et al, 2001), the successful transduction of signals through complex I blocks complex II and apoptosis. Conversely, in cells sensitive to TNF-mediated apoptosis, the signal activating NF-κB is not productive of $cFLIP_L$, and so caspase-8 becomes activated. These data are complemented by the recent observation that caspase-8 is not activated at the membrane associated TNFR1 signalling complex (Harper et al, 2003). Together, these data highlight the close relationship that exists between the NF-κB and apoptotic pathways. They also imply that $cFLIP_L$ is a key regulator in deciding whether a cell should commence programmed death.

1.4.4 Discovery of the FLIP family as inhibitors of apoptosis

The FLIP family was discovered in 1997 through data-base mining to identify viral genes containing DEDs and therefore related to apoptosis (Bertin et al, 1997; Thome et al, 1997; Hu et al, 1997a). The first FLIP genes were discovered within two oncogenic γ-herpesviruses and a human poxvirus associated with benign neoplasms of the skin: ORF71 of HVS (HVS-FLIP), ORF E8 of equine herpesvirus 2 (EHV-2) and ORF MC159L of molluscum contagiosum virus (MVC) respectively. The herpesvirus proteins are similar to the prodomain of caspase-8, consisting of two DED domains with an intervening short linker, while MC159L contains an extended C-terminus. Initial experiments transfected these viral FLIP proteins into cells to demonstrate protection against CD95- and TNFR1-induced apoptosis (Bertin et al, 1997; Thome et al, 1997; Hu et al, 1997a). Bertin et al, demonstrated E8 binding to caspase-8 and MC159L binding to FADD (Bertin et al, 1997). Hu et al, reported similar interactions and showed the protective effect of E8 and MC159L against TRADD and FADD, but not caspase-8 overexpression (Hu et al, 1997a). Finally, Thome et al, reported that E8, MC159L and HVS-FLIP are recruited to the DISC via interactions with FADD (Thome et al, 1997). In these experiments, co-expressed viral FLIP proteins were associated with an incomplete DISC that contained normal levels of CD95 and FADD, but reduced levels of procaspase-8 and activated caspase-8 (Thome et al, 1997). The viral FLIPs were proposed to protect infected cells from premature

apoptosis by preventing caspase-8 maturation, and therefore enhance lytic production of virus. Thome et al, identified ORF71 of KSHV as a FLIP homologue but did not perform functional studies with this protein (Thome et al, 1997).

1.4.5 Cellular FLIP

The cellular homologue, cFLIP, was subsequently identified (Irmler et al, 1997). It has also been termed CASH (caspase homologue) (Goltsev et al, 1997), casper (caspase-eight-related) (Shu et al, 1997), CLARP (caspase-like apoptosis-regulatory protein) (Inohara et al, 1997), FLAME-1 (FADD-like antiapoptotic molecule) (Srinivasula et al, 1997), I-FLICE (inhibitor of FLICE) (Hu et al, 1997b), MRIT (MACH-related inducer of toxicity) (Han et al, 1997) and usurpin (usurps caspase-8 and inhibits cell death) (Rasper et al, 1998). Several differentially spliced forms of cFLIP exist, but only two forms have been identified as proteins *in vivo*: short cFLIP ($cFLIP_S$) of 26 kDa and long cFLIP ($cFLIP_L$) of 55 kDa. $cFLIP_S$ is similar in structure to the herpesvirus FLIP proteins with an extended C-terminus of 20 amino acids. The N-terminus of $cFLIP_L$ is identical to that of $cFLIP_S$, but the C-terminus consists of a caspase-homologous domain that is catalytically inactive and contains cysteine and histidine residue substitutions within the enzymatic active site (Irmler et al, 1997; Rasper et al, 1998). In overall structure, $cFLIP_L$ is therefore similar to caspase-8 and -10. Like caspase-8 and -10, $cFLIP_L$ also contains an aspartic acid cleavage site (Asp-341) (Srinivasula et al, 1997) that is processed *in vivo* in the context of Fas-induced apoptosis (Scaffidi et al, 1999b; Krueger et al, 2001).

Both forms of cFLIP are recruited to the DISC and interfere with the function of caspase-8, although their mechanism of action is different. Like the viral FLIPs, $cFLIP_S$ is recruited by FADD and prevents the processing and release of active caspase-8 (Krueger et al, 2001). The role of $cFLIP_L$ is more controversial. $cFLIP_L$ binds directly to caspase-8 via DED and caspase domains (Han et al, 1997; Irmler et al, 1997; Srinivasula et al, 1997; Rasper et al, 1998), and both proteins are partially processed at the DISC (Scaffidi et al, 1999b; Krueger et al, 2001). Counterintuitively, overexpression of $cFLIP_L$ induced a cytocidal effect in some studies (Goltsev et al, 1997; Han et al, 1997; Inohara et al, 1997; Irmler et al, 1997; Shu et al, 1997).

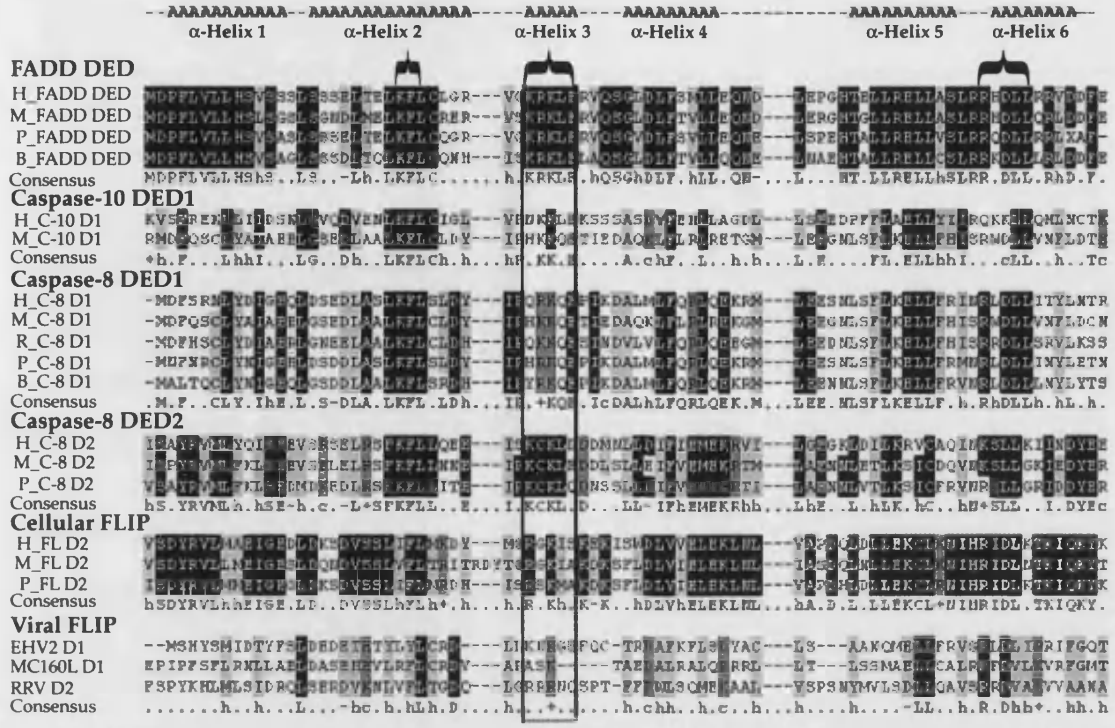
Furthermore, the phenotype of cFLIP$^{-/-}$ mice (Yeh et al, 2000) is strikingly similar to that of caspase 8$^{-/-}$ (Varfolomeev et al, 1998) and FADD$^{-/-}$ (Yeh et al, 1998) mice. These mice all died at E10.5-11.5 with impaired heart development and haemorrhaging, suggesting that cFLIP shares function with caspase-8 and FADD, rather than antagonising their activity. However, many studies have also demonstrated that in overexpression both forms of cFLIP were protective against apoptosis induced by DRs including Fas, TNFR1, TRAIL-R1, TRAIL-R2 and TRAMP (Goltsev et al, 1997; Irmler et al, 1997; Srinivasula et al, 1997; Schneider et al, 1997; Hu et al, 1997b; Rasper et al, 1998). These data are supported by the observation that MEFs from cFLIP$^{-/-}$ mice (deficient in cFLIP$_L$ and cFLIP$_S$) show increased sensitivity to CD95-induced apoptosis (Yeh et al, 2000), and that specific RNAi targeting of cFLIP$_S$ renders cells sensitive to TRAIL-mediated apoptosis (Siegmund et al, 2002). Although the majority of reports concur that cFLIP$_S$ is antiapoptotic, opinion remains divided over cFLIP$_L$. Perhaps the most conclusive evidence, to date, demonstrated that cFLIP$_L$ forms catalytically active heterodimeric complexes with caspase-8 (Micheau et al, 2002), and that physiological levels of cFLIP$_L$ enhance caspase-8 activation (Chang et al, 2002). Chang et al, also demonstrated that inhibition of apoptosis by cFLIP$_L$ only occurred at higher levels of expression that are sometimes associated with tumours.

cFLIP also activates NF-κB, a function for which the DEDs are required (Chaudhary et al, 2000; Hu et al, 2000; Kataoka et al, 2000). In Jurkat cells, Fas stimulation induced the recruitment of cFLIP$_L$ to the receptor where cFLIP interacted with TRAF1 and 2 and with kinases, RIP and Raf-1, leading to activation of NF-κB and Erk (Kataoka et al, 2000). The functional consequence was production of IL-2. Recently, cFLIP$_L$ was shown to bind p105 and inhibit its processing when expressed heterologously in 293T cells (Li et al, 2003). Both the DEDs and the caspase domain interacted with p105, although only the caspase domain could inhibit p105 processing. Furthermore, overexpression of p105 enhanced cFLIP$_L$-induced apoptosis and inhibited cFLIP$_L$-induced NF-κB activation (Li et al, 2003). This discovery adds a further layer of complexity to the relationship between FLIP proteins, apotosis and the NF-κB pathway.

1.4.6 vFLIP structure and homology

vFLIP is 188 amino acids in length, with an apparent molecular weight of 23 kDa. It is a bipartite molecule composed of two DEDs of approximately 90 amino acids each with an intervening linker domain. DEDs belong to the DD superfamily (Weber et al, 2001) that includes the DD, caspase recruitment domain (CARD) and pyrin domain families (Fairbrother et al, 2001). These domains form strong homophilic interactions and play a pivotal role in apoptotic signal transduction by recruiting adaptor and effector molecules. They are essential to the formation of both the DISC and apoptosome. Although members of the DD superfamily share only 5-25% sequence similarity, structural studies have revealed a common fold: six antiparallel, amphipathic α-helices (Huang et al, 1996; Liepinsh et al, 1997; Eberstadt et al, 1998; Sukits et al, 2001; Kaufmann et al, 2002). The majority of DEDs share a conserved RXDL motif within the α-helix 6 (Fig. 1.8), which is essential for protection against DR-mediated apoptosis by MC159L (Garvey et al, 2002). Kaufmann et al, observed significant diversity within α-helix 3 (Fig. 1.8), and proposed a sub-division of DEDs into two classes (Kaufmann et al, 2002). Class I possess basic residues in α-helix 3 and include the DED of FADD, both DEDs of caspase-8 and the C-terminal DED of cFLIP. In class II, the α-helix 3 is shortened or absent. Members of class II include most of the viral FLIP DEDs and the N-terminal DED of cFLIP (Kaufmann et al, 2002). Mutations within α-helix 3 of the DED of FADD influence the recruitment of cFLIP and caspase-8 to the DISC (Kaufmann et al, 2002). Both DEDs of vFLIP fall into class II (Fig. 1.8). A predicted model for the second vFLIP DED is shown in Fig. 1.9.

A) Class I DEDs



B) Class II DEDs

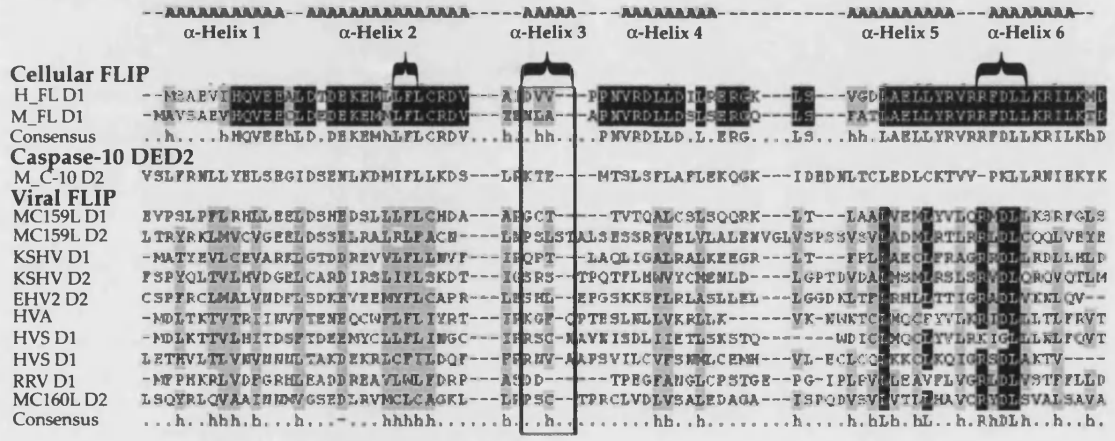


Figure 1.8. Structure-based alignment of DEDs.

Class I DEDs (A) and class II DEDs (B) are aligned to demonstrate key features (taken from Kaufmann et al, 2002). The heterogeneous nature of helix 3 (boxed) is shown. Positions of FADD-DED α-helices are indicated. Residue F25, the basic stretch and the RXDL motif are indicated by a brace. Consensus sequences are shown (h: hydrophobic, +: basic, -: acidic, c: charged). H, Homo sapiens; M, Mus musculus; P, Sus scrofa; B, Bos taurus; R, Rattus norvegicus.

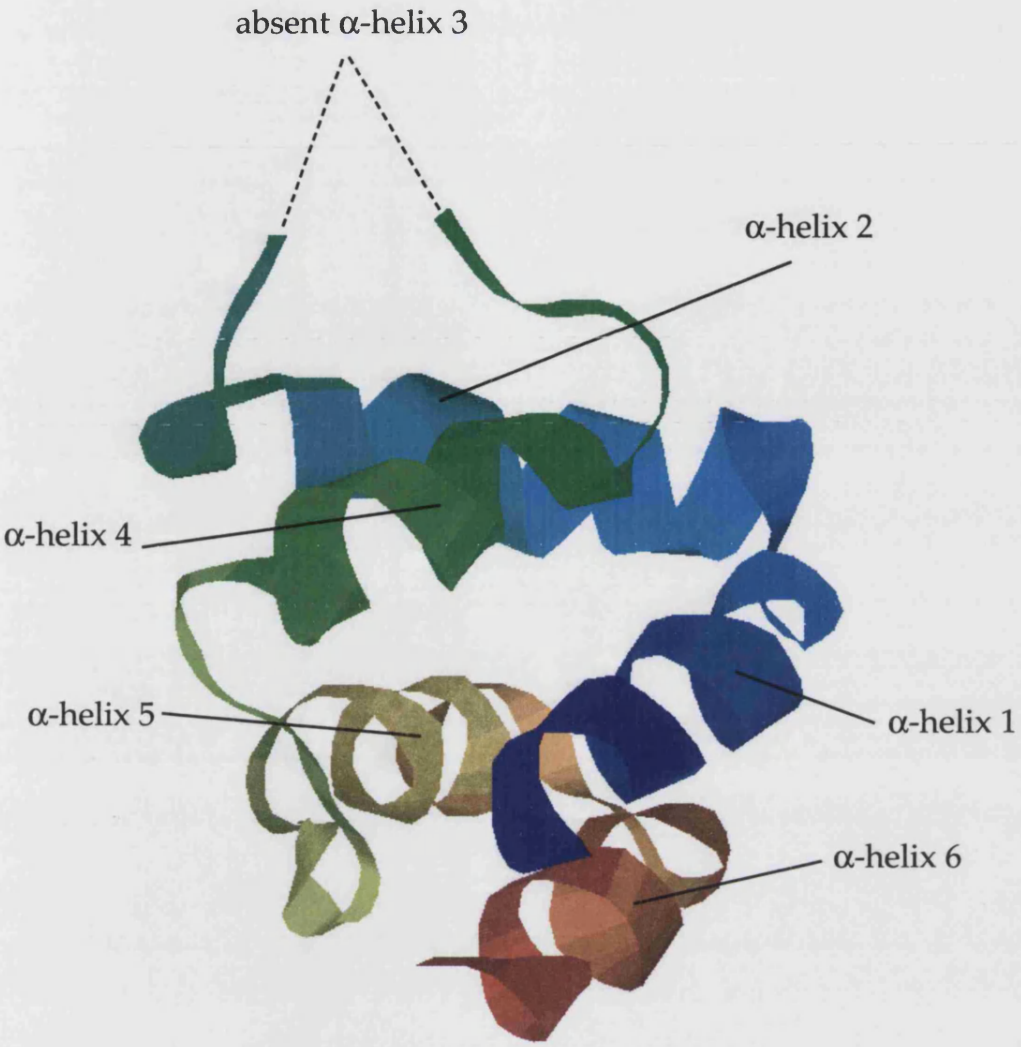


Figure 1.9. Model of vFLIP second DED.

The structure of vFLIP second DED was predicted according to 3d-pssm (Kelley et al, 2000). This method uses structural alignment of homologous proteins with known three-dimensional structure to predict structural equivalence for residues in a polypeptide of unknown structure. This predicted structure was based upon that of the DD of FADD (Eberstadt et al, 1998), with which it shares significant homology. Each α-helix is shown in colour and labelled. However, 3d-pssm was unable to predict structure for the region equivalent to α-helix 3 in the FADD-DD. This observation may support primary sequence analysis demonstrating significant heterogeneity within the a-helix 3 of different DEDs (Kaufmann et al, 2002).

The family of FLIP proteins has grown as increasing numbers of viral genomic sequences have been published. At present, viral FLIP proteins are exclusive to the γ-2-herpesvirus lineage, with the notable exception of MCV (Senkevich et al, 1996). The independent acquisition of a FLIP gene by this unrelated oncogenic virus points to the importance of FLIP. To date, the set of herpesviruses encoding a viral FLIP includes: KSHV (Russo et al, 1996; Neipel et al, 1998), HVS (Albrecht et al, 1992), EHV-2 (Telford et al, 1995), bovine herpesvirus 4 (BoHV4) (Zimmermann et al, 2001), herpesvirus ateles (HVA) (Albrecht 2000) and rhesus rhadinovirus (RRV) (Alexander et al, 2000). The family Herpesviridae currently boasts approximately 120 members, but it is likely that this represents only a fraction of the total number of herpesviruses in existence (Davison 2002). As more herpesviruses are discovered and sequenced, it seems certain that more viral FLIP proteins will also be discovered. Interestingly, EBV does not encode a FLIP protein despite being closely related to KSHV. It is therefore likely that the acquisition of FLIP is a relatively recent event in herpesvirus evolution. Furthermore, vFLIP is not found in every γ-2-herpesvirus, since murine herpesvirus-68 (MHV-68) (Virgin et al, 1997) and alcelaphine herpesvirus-1 (AHV) (Ensser et al, 1997) do not encode a FLIP gene. It is not clear whether these viruses diverged from the other γ-2-herpesviruses before the acquisition of FLIP, or whether they acquired the gene from a common ancestor and subsequently lost it.

1.4.7 The function of vFLIP

vFLIP is expressed from a bicistronic mRNA by means of an IRES that is located within the vcyclin ORF (Section 1.2.7.1). IRES elements are found in a few eukaryotic mRNAs that encode growth factors (e.g. VEGF), oncogenes (e.g. c-myc) and an inhibitor of apoptosis (XIAP) (Bieleski et al, 2001). The IRES elements seem to ensure efficient translation of mRNA throughout the cell cycle, and particularly during G_2/M phase when there is a general loss of cap-dependent translation (Bonneau et al, 1987; Huang et al, 1991). It is suggested that IRES-mediated expression of vFLIP guarantees protein expression at times during the cell cycle when translation is limited (Bieleski et al, 2001). The fact that KSHV has acquired such a

mechanism to regulate the expression of vFLIP points to an important role for this protein in KSHV infection.

By analogy with other viral FLIPs, vFLIP has been suggested to block DR-mediated apoptosis (Thome et al, 1997). Indeed, overexpression of vFLIP in HeLa cells blocks procaspase-8 cleavage and reduces caspase-3 and caspase-8 activity (Belanger et al, 2001). These data are consistent with the ability of vFLIP to promote tumour growth when expressed in a Fas-sensitive B cell lymphoma line injected into immunocompetent mice (Djerbi et al, 1999). Interestingly, the growth advantage conferred by vFLIP was not apparent when cells were injected into immunodeficient mice, suggesting that vFLIP protects against immune rejection of KSHV-infected cells (Djerbi et al, 1999). Similarly, experiments using a different mouse model demonstrated that cFLIP can directly mediate escape from T cell immunity *in vivo* (Medema et al, 1999). These experiments defined a new class of tumour progression factor (Djerbi et al, 1999), implying a role for vFLIP in KSHV-mediated oncogenesis.

More recently, vFLIP has been implicated in the regulation of transcriptional pathways. Chaudhary et al, first demonstrated the activation of NF-κB driven reporter constructs in 293T cells by vFLIP, but not E8 or MC159L (Chaudhary et al, 1999). The level of NF-κB activation was modest (7-fold) in this initial report. However, using less DNA, others and I have routinely demonstrated >100-fold activation of NF-κB by vFLIP (An et al, 2003b)(Fig. 4.5). Moreover, An et al, contradict the finding by Chaudhary et al, that vFLIP does not activate the JNK/AP1 pathway (An et al, 2003b). The activation of both NF-κB and JNK/AP1 pathways by vFLIP was shown to drive cIL-6 expression (An et al, 2003b) in synergy with LANA (Section 1.2.7.2). In view of the essential function cIL-6 is thought to play in KSHV-associated neoplasms (Section 1.2.8.3), this observation alone implies an important role for vFLIP in KSHV pathobiology. Subsequently, Chaudhary and colleagues have shown that vFLIP-induced NF-κB activation protects against growth factor withdrawal-induced apoptosis (Sun et al, 2003a), although vFLIP had no significant effect against TNF-mediated apoptosis (Matta et al, 2002; Sun et al, 2003a). This is surprising, in view of previous observations that HVS-FLIP, E8 and MC159L do inhibit DR-mediated apoptosis, but afford no protection against growth factor withdrawal-

induced apoptosis (Thome et al, 1997). The inference being that vFLIP may have a different biological function to other FLIP proteins, both viral and cellular.

Chaudhary and colleagues have recently shown that vFLIP can transform Rat-1 and Balb/3T3 cells (Sun et al, 2003b). Expression of vFLIP, but not E8 or MC159L, in these cells led to loss of contact inhibition, growth in soft agar and formation of tumours in nude mice (Sun et al, 2003b). These features of transformation were abrogated by use of NF-κB inhibitors including lactacystin, aspirin, phenylarsine, arsenic trioxide and the IκBα super-repressor, implying an essential role for NF-κB in vFLIP-induced transformation (Sun et al, 2003b). Furthermore, using site-directed mutagenesis, the transforming efficiency of vFLIP mutants was found to correlate with their ability to activate NF-κB (Sun et al, 2003b). At a molecular level, the ability of vFLIP to activate NF-κB has now been partially explained. Concurrent with the work presented in this thesis, it was demonstrated that vFLIP interacts with and activates the IKK complex when expressed in a non-small-cell lung carcinoma cell line (Liu et al, 2002). Using cells with single gene deletions, the NF-κB-inducing activity of vFLIP has been shown to depend upon all three IKK subunits (Matta et al, 2003). In support of these data, it is shown in Chapters 3 and 4 that vFLIP contacts IKKγ directly to activate IKK, and that the majority of endogenous vFLIP is associated with IKK in a KSHV-infected PEL cell line. Interestingly, a number of other DED-containing proteins have been shown to activate NF-κB including caspase-8, caspase-10, cFLIP and FADD (Chaudhary et al, 2000; Hu et al, 2000; Kataoka et al, 2000; Shikama et al, 2003). At present it is not clear how these proteins activate NF-κB, and there is no evidence to suggest that they activate NF-κB via an interaction with IKKγ.

NF-κB is a potent mediator of apoptosis through the regulated expression of proteins that control the apoptotic threshold (Section 1.4.3). Activation of NF-κB may therefore account for the anti-apoptotic properties of vFLIP (Djerbi et al, 1999; Belanger et al, 2001). Perhaps the most persuasive evidence that NF-κB prevents apoptosis was the observation that ablation of TNFα (Doi et al, 1999) or TNFR1 (Rosenfeld et al, 2000; Alcamo et al, 2001) could rescue the lethal phenotype of RelA-deficient mice (Beg et al, 1995). These mice die due to extensive hepatic

apoptosis, and these observations suggest that RelA protects against TNFα-mediated apoptosis. Furthermore, MEFs derived from $RelA^{-/-}$ (Beg et al, 1996) and $IKK\beta^{-/-}$ mice (Tanaka et al, 1999; Li et al, 1999b; Li et al, 1999d) show increased sensitivity to TNFα-mediated apoptosis *in vitro*. Expression of the IκBα super-repressor both *in vitro* (Wang et al, 1996) and *in vivo* (Wang et al, 1999) reverses resistance to apoptosis induced by chemotherapeutic agents in some cancers, suggesting a role for NF-κB in regulating other forms of apoptosis. Interestingly, vFLIP-mediated resistance to growth factor withdrawal-mediated apoptosis was dependent on NF-κB (Sun et al, 2003a). Anti-apoptotic genes regulated by NF-κB include members of the anti-apoptotic Bcl-2 family (Bcl-2, Bcl-X_L, Bfl-1), the IAP family (XIAP, cIAP1, cIAP2) and cFLIP (Section 1.4.3) (Burstein et al, 2003).

Significantly, NF-κB activity is high in PEL cell lines (Keller et al, 2000; Liu et al, 2002) and primary tumour specimens (Keller et al, 2000). Furthermore, inhibition of NF-κB using the specific inhibitor of IκBα phosphorylation, Bay11-7082, triggers apoptosis in KSHV-infected PEL cells (Keller et al, 2000), suggesting that NF-κB activity is essential for their survival. In addition to vFLIP, KSHV is known to encode three proteins with the potential to activate NF-κB: vGPCR, K1 and K15 (Table 1.2). However, vGPCR and K1 are lytic proteins, and K15 activated NF-κB only weakly (4-fold) in overexpression (Brinkmann et al, 2003). As the only potent activator of NF-κB expressed in latently infected PEL cells, vFLIP is the most likely molecular source of the NF-κB activity observed in latent KSHV infection.

1.5 Aims of this study.

vFLIP is one of seven known latent genes expressed by KSHV that regulate viral latent infection and may be essential for KSHV-mediated cell transformation. At the time this study was initiated, published data relating directly to the function of vFLIP were scarce. One study described NF-κB activation by vFLIP in transient overexpression (Chaudhary et al, 1999), and another study defined vFLIP as a tumour progression factor that inhibited Fas-mediated apoptosis (Djerbi et al, 1999). Other viral FLIP proteins block apoptosis directly by preventing caspase-8 maturation. Despite a lack of direct evidence, vFLIP was therefore generally considered to function as a direct inhibitor of DISC activity.

The scope of this project was to study the molecular interactions of vFLIP in KSHV-infected PEL cells, and to pursue the pathways of cellular signal transduction affected by vFLIP. This thesis therefore sets out to describe in detail the molecular characteristics of vFLIP. Chapter 3 describes the discovery that vFLIP associates with and activates IKK in PEL cells. Chapter 4 focuses on the direct interaction between vFLIP and IKKγ. In Chapter 5, it is suggested that vFLIP stimulates the alternative pathway of NF-κB activation. Finally, in Chapter 6 these results are brought together to update the current understanding of vFLIP function and to suggest how vFLIP may contribute to the pathogenesis and cell transformation associated with KSHV infection.

CHAPTER 2

Materials and Methods

2.1 Buffers and solutions.

Deoxynucleotide triphosphate mix (dNTPs)	100 mM deoxyadenosine triphosphate (dATP), deoxythymidine triphosphate (dTTP), deoxyguanosine triphosphate (dGTP) and deoxycytidine triphosphate (dCTP)
6x DNA loading buffer	60 mM Tris pH 7.4, 6 mM ethylenediaminetetraacetic acid (EDTA) (pH 8.0), 30% (v:v) glycerol, 0.25% (w:v) Orange G
Glutathione elution buffer	10 mM reduced glutathione in 50 mM Tris pH 8.0
Luria-Bertani (LB) agar	1% (w:v) bacto typtone, 0.5% (w:v) bacto yeast, 0.5% (w:v) sodium chloride (NaCl), pH 7.0 with 15g/L bacto-agar
Luria-Bertani (LB) broth	1% (w:v) bacto typtone, 0.5% (w:v) bacto yeast, 0.5% (w:v) NaCl, pH 7.0
Kinase reaction buffer	20 mM HEPES pH 7.6, 50 mM NaCl, 10 mM magnesium chloride (MgCl), 2 mM Dithiothreitol (DTT), 20 μM ATP, 0.1 mM sodium vanadate (Na_3V0_4), 20 mM β-glycerophosphate (βGP) and protease inhibitor mix (PIM) (Roche)
Kinase wash buffer	20 mM HEPES pH 7.6, 50 mM NaCl, 20 mM βGP, 1 mM Na_2VO_4, 0.5 mM DTT, 1 mM phenylmethylsulfonyl fluoride (PMSF) and PIM
Non-denaturing polyacrylamide gel	4% (v:v) acrylamide (29 acrylamide: 1 bis), 0.25x TBE, polymerised with 0.06% (w:v) ammonium persulphate (APS) and 0.16% (v:v) tetramethylethylenediamine (TEMED)
Nonidet P-40 (NP40) lysis buffer	20 mM Tris pH 7.5, 150 mM NaCl, 0.2% (v:v) NP40, 1 mM EDTA, 1 mM ethylenedioxy nitrilotetraacetate (EGTA), 1 mM DTT, 20 mM sodium fluoride (NaF), 1 mM sodium pyrophosphate ($Na_4P_2O_7$), 1 mM Na_3VO_4, 5% (v:v) glycerol, 1 mM PMSF and PIM
Nuclear lysis buffer	20 mM HEPES pH 7.6, 0.2 mM EDTA, 0.1 mM EGTA, 25% (v:v) glycerol, 0.42 mM NaCl, 1 mM DTT, 20 mM NaF, 1 mM $Na_4P_2O_2$, 1 mM Na_3VO_4, 1 mM PMSF and PIM
2x Parker buffer	8% (v:v) ficoll, 40 mM Hepes pH 7.9, 100 mM KCl, 2 mM EDTA, 1 mM DTT
Phosphate-buffered saline (PBS)	137 mM NaCL, 2 mM potassium chloride (KCl), 10 mM sodium hydrogen phosphate (dibasic), 2 mM potassium hydrogen (dibasic), pH 7.4
Polyacrylamide resolving gel	12% (v:v) acrylamide (37.5 acrylamide: 1 bis), 125 mM Tris pH 8.8, 0.1% (w:v) sodium dodecyl sulphate (SDS), polymerised with 0.05% (w:v) APS and 0.1% (v:v) TEMED
Polyacrylamide stacking gel	5% (v:v) acrylamide (37.5 acrylamide: 1 bis), 125 mM Tris pH 6.8, 0.1% (w:v) SDS, polymerised with 0.05% (w:v) APS and 0.1% (v:v) TEMED
6x Protein sample buffer	6% (w:v) SDS, 125 mM Tris pH 6.8, 36% (v:v) glycerol, 15% (v:v) β-mercaptoethanol with bromophenol blue

Radioimmuno-precipitation (RIPA) lysis buffer	150 mM NaCl, 50 mM Tris pH 7.5, 1% (v:v) Triton, 0.5% (w:v) sodium deoxycholate (DOC), 0.1% (w:v) SDS, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 20 mM NaF, 1 mM $Na_4P_2O_7$, 1 mM N_3VO_4, 1 mM PMSF and PIM
SDS-polyacrylamide gel electrophoresis (PAGE) running buffer	25 mM Tris pH 8.5, 200 mM glycine, 0.1% (w:v) SDS
Transformation buffer	250mM PIPES, 2.5mM calcium chlorohydrate ($CaCl_2.2H_2O$), 60 mM KCl, adjusted to pH 6.7 using potassium hydroxide (KOH) before addition of 55 mM manganese chloride ($MnCl_2$)
1x Tris-acetate-EDTA (TAE)	40 mM Tris pH 7.8, 20 mM sodium acetate, 1 mM EDTA
5x Tris-borate-EDTA (TBE)	450 mM Tris pH 8.0, 450 mM boric acid, 10 mM EDTA

Table 2.1. Constituents of buffers and solutions

2.2 Subcloning and plasmid preparation.

Subcloning is the process whereby a specific fragment of DNA is transferred from one plasmid to another. The methods utilised in this process are described in Section 2.2.

2.2.1 Preparation of heat-shock competent *E. coli.*

1ml of an overnight culture of E. coli HB101 or FB810 in LB (GibCoBRL) was subcultured into 100ml of fresh LB and shaken for 2h at 37°C. The culture was put on ice for 10 min, and then pelleted at 4°C and the supernatant discarded. The pellet was resuspended in 30ml ice-cold transformation buffer. The bacteria were pelleted and resuspended in 10ml ice-cold transformation buffer containing 10% DMSO and then frozen at -80°C in aliquots.

2.2.2 Transformation of heat-shock competent *E. Coli.*

10-50ng of plasmid were incubated on ice with 50µl of heat shock competent *E. coli* HB101 or FB810 for 5 min. The bacteria were then shocked for 90s at 42°C and cooled on ice. 600µl warm LB was added and the bacteria were incubated for 30 min

at 37°C before pelleting. Pellets were resuspended in 50µl and plated onto LB-agar containing the appropriate antibiotic and incubated overnight at 37°C.

2.2.3 Plasmid DNA mini-preps.

To obtain small quantities (5-25 µg) of plasmid DNA, mini-preps were produced from 1-5ml overnight cultures of transformed bacteria using a Concert™ Rapid Plasmid DNA Mini-prep kit (GibCoBRL) as per the manufacturer's instructions.

2.2.4 Plasmid DNA midi-preps.

Larger quantities (200µg) of pure plasmid DNA were extracted from a 100ml bacterial culture. Midi-preps were produced from this culture using the Plasmid Midi Kit (Qiagen) as per the manufacturer's instructions. The concentration of purified DNA was calculated from the UV absorbance at 260 nm using a UV spectrophotometer (Camlab). An absorbance of $1cm^{-1}$ was taken to be equivalent to 50 $\mu g.ml^{-1}$ DNA.

2.2.5 Polymerase chain reaction (PCR) amplification.

PCR was performed to generate each IKKγ fragment from an expressed sequence tag, and the fragment encoding IκBα wild-type from pGEX-KT-IκBα, and the fragment encoding IκBα S32A/S36A from RSV IκBα MSS using a Hybaid thermal cycler. Primers are listed in Table 2.2. The constituents of each reaction were:

DNA (25ng)	2 µl
Primers (100µM)	2 µl each
Takara PCR buffer	5 µl
dNTPs	1 µl
Takara TaqEx	0.5 µl

Distilled water 37.5 µl

The product yield for all PCR reactions to amplify IKKγ fragments was poor. Following optimisation, 4% DMSO was added to these PCR reactions to enhance yield.

The reaction conditions were:

1. 94°C 2 min
2. 94°C 30 Sec
3. 58°C 25 Sec
4. 72°C 30 Sec
5. Steps 2-4 an additional 30 times
6. 72°C 5 min

Amplified fragement	**Forward (F) and reverse (R) primer sequence**
IKKγ 1-150	F: GCGCGGATCCATGAATAGGCACCTCTGGAA R: GCGCCTCGAGTCAGAGCAAGGACGTGCAGCTG
IKKγ 1-272	F: GCGCGGATCCATGAATAGGCACCTCTGGAA R: GCGCCTCGAGTCACTCCTCGGCCTGCTGGA
IKKγ 1-419	F: GCGCGGATCCATGAATAGGCACCTCTGGAA R: GCGCCTCGAGCTACTCAATGCACTCCATG
IKKγ 150-272	F: GCGCGGATCCCTCGGGGAGCTGCAGGAG R: GCGCCTCGAGTCACTCCTCGGCCTGCTGGA
IKKγ 150-419	F: GCGCGGATCCCTCGGGGAGCTGCAGGAG R: GCGCCTCGAGCTACTCAATGCACTCCATG
IκBα 1-54	F: GCGCAAGCTTAATGTTCCAGGCGGCCGAG R: GCGCAGATCTTGAGAGGCGGATCTCCTGCA

Table 2.2. PCR primers.

2.2.6 Restriction enzyme digests.

Restriction enzyme digests were used to create sticky ends for ligation of PCR products into plasmids, and to subsequently screen for correct insert and orientation. All restriction enzymes were purchased from Promega and used as per manufacturer's instructions. The reaction was stopped by the addition of the appropriate volume of 6x DNA loading buffer.

2.2.7 Agrose gel electrophoresis.

The products of PCR amplification and restriction enzyme digests were separated on the basis of size by electrophoresis on a 1% agrose gel containing 0.5μg/ml ethidium bromide in TAE. When necessary, specific bands were excised with a scalpel and DNA subsequently extracted using a Concert™ Rapid DNA fragment gel purification kit (GibCoBRL) as per the manufacturer's instructions.

2.2.8 Ligations.

Using a weight ratio of 1 vector:4 insert, 5μl of vector plus insert were mixed with 5μl of Takara DNA ligase solution for a final volume of 10μl and incubated at 16°C for 2h. Ligation solutions were transformed by heat shock into *E. coli* and plated onto LB-agar (Section 2.1.3). Single colonies were picked and plasmid DNA was prepared by mini-prep (Section 2.1.4) for screening by restriction enzyme digest and then sequencing by I. Gerrard at the Windeyer Institute sequencing service.

2.3 Plasmids.

2.3.1 Mammalian expression plasmids.

PCR primers (Table 2.2) were used to amplify and subclone IKKγ fragments into pcDNA4 (Invitrogen) (Sections 2.2; 4.2). pcDNA3.1-HA-p105 and pcDNA3.1-Myc-p100 were kind gifts from S. Ley (Mill Hill, UK). The pGL2 promoter vector (Promega) with 3 copies of an NF-κB concensus binding site upstream of firefly luciferase was obtained from S. Wotton (UCL, UK). The plasmid RSV IκBα MSS super-repressor (Section 1.3.3) was a kind gift from N. Perkins (Dundee, UK) and was used to subclone the N-terminal fragment of IκBα-S32A/S36A into pGEX-KT (Section 2.3.2).

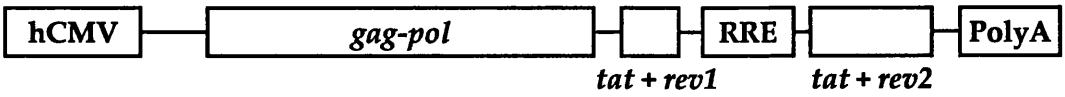
2.3.2 *E. Coli* expression plasmids.

The plasmid pGEX-KT-IκBα was a kind gift from N. Perkins (Dundee, UK). To obtain GST-IκBα fusion proteins containing only the N-terminal 1-54 amino acids of IκBα wild-type and IκBα-S32A/S36A, PCR primers (Table 2.2) were used to amplify and subclone this domain into pGEX-KT. GST-fusion IKKγ mutants were constructed by PCR amplification and subcloning of fragments from pcDNA4-IKKγ.

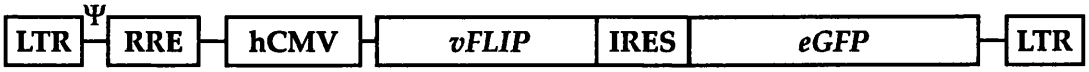
2.3.3 HIV-1 based plasmids

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 packaging plasmid pHCMVΔR8.1 expresses *gag*, *pol*, *tat*, and *rev* and does not express the accessory genes *vif*, *vpr*, *vpu* or *nef* (Zufferey et al, 1997) (Fig. 2.1). The vector plasmid pHR'-hCMV-eGFP contains the reporter gene, enhanced green fluorescent protein (eGFP), under the control of the human cytomegalovirus (hCMV) immediate early promoter. This plasmid was modified within our laboratory to express both vFLIP and eGFP from the internal ribosome entry site (IRES) of encephalomyocarditis virus (EMCV) (Fig. 2.1). The pMD-G plasmid encodes the vesicular stomatitis virus G (VSV-G) envelope glycoprotein (Zufferey et al, 1997) (Fig. 2.1). The Tax expression plasmid pHR'-hCMV-Tax-IRES-eGFP was a gift from A. Godfrey (UCL, UK).

pHCMVΔR8.91 Packaging plasmid



pHR'-hCMV-vFLIP-IRES-eGFP Vector plasmid



pMD-G Envelope plasmid

Figure 2.1. HIV packing, vector and envelope plasmids.

Transcription of *gag-pol* in pHCMVΔR8.91 is controlled by hCMV. pHCMVΔR8.91 also encodes *tat* and *rev*. Transcription of the pHR'hCMV-vFLIP-IRES-eGFP vector transcript is controlled by the LTR at the 5' end and terminates in the 3' LTR. The transgene cassette, encoding vFLIP and eGFP, is controlled by the internal hCMV promoter. The positions of the packaging signal (Ψ) and rev responsive elements (RRE) are shown. Transcription of the *VSV-G* envelope is also controlled by hCMV. Regions encoding protein products within the mRNA are shown in grey and regions encoding *cis*-acting elements in white.

2.4 Cell culture techniques.

2.4.1 Cell lines, cell culture and reagents.

Cell culture medium and serum were obtained from GibCoBRL. Human embryonic kidney (HEK) 293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% foetal calf serum (FCS), penicillin and streptomycin in a 10% CO_2 humidified incubator at 37°C. Cells were split 1:10 every 3-4 days to ensure optimal cycling. Human B cell lines included the KSHV-transformed and EBV negative PEL cell line, BC3 (Arvanitakis et al, 1996)(Section 1.2.5.2), and the anablastic diffuse large B cell lymphoma cell line, DEL (Barbey et al, 1990). Human $CD3^{+ve}$ Jurkat 3T8 T cells and their IKKγ-deficient derivative, 8321 (He et al, 2002), were obtained from S. Ley (Mill Hill, UK) by kind permission from A. Ting (New York, USA). All non-adherent cell lines were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium with 10% FCS, penicillin and streptomycin in a 5% CO_2 humidified incubator at 37°C, and cell density was kept within the range $1x10^5$-$1x10^6$ cells/ml. 293T and BC3 cells were treated with 0.5μM Geldanamycin (GA) (Calbiochem) dissolved in DMSO or an equal volume of DMSO in serum free medium for 16h or 48h before lysis as described in Chapter 3. 293T cells were treated with 10ng/ml TNFα (Sigma) before lysis as described in Sections 3.5 and 4.4.

2.4.2 Lipofectamine-mediated transfection.

Plasmid DNA was introduced into 293T cells using lipofectamine (GibCoBRL). Cells were plated at $1x10^6$ cells per well in 6-well plates 24h before transfection. On the day of transfection, 16μl of plasmid DNA (mass is stated in results chapters) was added to 10μl of OptiMEM and 6μl of lipofectamine for each well. This mixture was incubated at 24°C for 30 min while cells were washed twice and left in 0.8ml of OptiMEM (GibCoBRL). A further 200μl of OptiMEM was added to the cocktail containing DNA and lipofectamine and the final volume of 232μl was mixed by pipetting and

then added dropwise to each well. Cells were incubated for 6h at 37°C before the transfection mixture was removed and replaced with DMEM and 10% FCS.

2.4.3 Virus production and harvesting.

HIV-1 based vectors, referred to as lentivirus, were used to transduce 293T, DEL, 3T8 and 8321 cell lines. Lentivirus was produced using a three plasmid transient transfection system as described previously (Naldini et al, 1996; Zufferey et al, 1997) (Fig. 2.1). 24h before transfection, $1x10^7$ 293T cells were plated into 20cm plates. Using a weight ratio of 3:2:1 of vector to packaging to envelope plasmids (Section 2.3.3), cells were transfected exactly as described in Section 2.4.2, except that all volumes were scaled up by a factor of 10. After 6h incubation at 37°C, the cells were washed and grown in OptiMEM for 48h at 32°C for optimal virus recovery. Supernatants were harvested, passed through a 45-μM-pore-size filter, and concentrated by low-speed centrifugation using a 100 kDa cut-off filter column (Millipore). The virus was then aliquoted and stored at -80°C prior to use.

2.4.4 Titrating viral supernatants and infecting tissue culture cells with viral supernatants.

For titration of viral supernatants, 293T cells were plated at $1x10^5$ per well in 24-well plates 24h before infection. On the day of infection, virus supernatant was serially diluted in OptiMEM and added to wells. The cells were incubated overnight. The virus was then washed off the cells and replaced with DMEM and 10% FCS. Cells were cultured for 7 days post infection, until infected cells displayed uniform cytoplasmic eGFP. Uninfected cells were passed through a FACSCaliber and analysed using CELL QUEST software (Becton Dickinson, Franklin Lakes, USA) to determine side vs. forward scatter characteristics and select the region in which live cells could be found. For each infection, $1x10^4$ cells in this region were recorded and analysed for eGFP expression. The side scatter vs. green fluorescence (FL-1) plot of uninfected cells determines the region in which cells not expressing eGFP fall. A gate was placed in FL-1 to record the percentage of cells with higher fluorescence than the uninfected controls. Virus titre (infectious units/ml) was calculated from the

percentage of cells infected and the number of cells per well on the day of infection using the equation: (%-infected cells x number of cells at infection / 100) x dilution factor.

293T cells were infected for experimental purposes exactly as described above using multiplicity of infection (MOI) as stated in Fig. 3.6. For infection of DEL and Jurkat cell lines, $5x10^4$ cells were pelleted and resuspended in 1ml OptiMEM containing virus at an MOI of 20. After 6h cells were washed and cultured as described. For all cells, transduction efficiency was measured exactly as described above for 293T cells.

2.4.5 Cell viability assays.

The viability of BC3 cell populations was measured directly by haemocytometer using a light microscope to assess morphology and light-diffracting properties of each cell. Data were collected in triplicate and average values plotted with error bars calculated as the standard error of the mean (SEM). For annexinV/propidium-iodide binding assays, $1x10^6$ cells were washed once in cold phosphate buffered saline (PBS) before staining with TACS™ AnnexinV-FITC Apoptosis detection kit (R&D systems) as per the manufacturer's instructions. Analysis was performed by FACScan using CELL QUEST software. Side scatter vs. forward scatter was used to gate the region containing live cells, and $1x10^4$ cells within this region were recorded to determine the percentage of annexinV (FL-1) and propidium (FL-2) staining.

2.5 Preparation of GST-fusion proteins in *E. coli.*

The pGEX-KT-IκBα, pGEX-KT-IκBα-S32A/S36A and pGEX-KT-IKKγ expression constructs were transformed into *E. coli*, FB810, which contain an accessory plasmid expressing lysozyme. Cultures were grown to an optical density (OD) of 0.6 before induction with 0.1mM isopropyl-D-thiogalactopyranoside for 3h at 37°C. The cells were washed twice in ice-cold PBS and pelleted. Pellets were frozen at -20°C and thawed to release lysozyme by resuspension in lysis buffer (PBS supplemented with 0.2% NP40 and PIM). The suspension was sonicated for 30 sec and incubated at 4°C

for 30 min before centrifugation. The soluble extract was incubated with glutathione-sepharose 4B matrix (Amersham) for 30 min at room temperature. The matrix was pelleted and washed three times with PBS. Finally, GST-IKKγ mutant-proteins bound to the matrix were used directly in pull-down assays (Section 2.6.4), while GST-IκBα fusion proteins were eluted from the matrix using glutathione elution buffer, assayed for protein concentration (Section 2.6.2) and stored in aliquots at -80°C prior to use.

2.6 Analysis of mammalian cell extracts.

2.6.1 Preparation of cell extracts.

Cells were washed in PBS, pelleted and resuspended in either RIPA lysis buffer or NP40 lysis buffer as stated in results chapters. The suspension was incubated at 4°C for 30 min before insoluble material was removed by centrifugation at 4°C. At this point aliquots were removed for protein assay (Section 2.6.2). The supernatants were either used as described below, or the appropriate quantity of 6x protein sample buffer was added. These samples were then heated to 95°C for 4 min and stored at -80°C until required.

2.6.2 Protein assay.

Total protein per sample was estimated using the Bio-Rad Protein Assay based on the method of Bradford (Bradford 1976). This method is compatible with buffers containing 0.1% SDS. Sufficient Bio-Rad dye reagent was diluted 1:5 in H_2O. 5µl and 10µl of each sample were resuspended in 1ml of the diluted dye, vortexed and incubated at 24°C for 5 min. Total protein content was then determined by measuring the optical density (OD) at 595nm and comparison with BSA protein standard (Promega).

2.6.3 Identification of proteins interacting with vFLIP by mass spectrometry analysis.

2.6.3.1 Large-scale immunoprecipitation.

The anti-vFLIP 6/14 monoclonal rat antibody (Low et al, 2001), and control rat IgG were covalently coupled to NHS-activated sepharose 4B resin (Amersham) for batch immunoprecipitation. The antibodies were first concentrated to 4mg/ml by low-speed centrifugation using 100 kDa cut-off filter columns (Millipore). 1mg of antibody was coupled to 0.5ml resin in an equal volume of coupling buffer (0.2M $NaHCO_3$, 0.5M NaCl, pH 8.3) by incubation overnight at 4°C. The resin was washed and deactivated by alternating washes in buffer A (0.5M ethanolamine, 0.5M NaCl, pH 8.3) and buffer B (0.1M acetate, 0.5M NaCl, pH4); two washes in A, then two in B, repeated three times. Cell extract was prepared from $1x10^{10}$ BC3 cells and divided equally between vFLIP and control resins for incubation at 4°C for 2hr. The resin was washed three times in high salt lysis buffer (lysis buffer with 500mM NaCl) and 100µl of 1x 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 min. The samples were then divided 9:1 between two 12% polyacrylamide gels (Fig. 3.1). The gel containing 90% of the sample was stained with Colloidal Blue Coomassie staining kit (Invitrogen) as per the manufacturer's instructions. The gel containing 10% of the lysate was stained using the Shevchenko-Hochstrasse method of silver staining.

2.6.3.2 In-gel digest.

Protein bands of interest were excised from Coomasssie stained gels. SDS and Coomasssie were extracted from the gel slices with solution A (200mM ammonium bicarbonate, 50% acetonitrile). The gel slices were reduced in DTT (20mM DTT in solution A) and washed in solution A. Cysteine residues in the proteins were alkylated in iodoacetamide (5mM iodoacetamide in solution A) and gel slices were washed in solution A. The gel slices were dehydrated in acetonitrile and sent for mass spectrometry analysis to our collaborators at the National Institute of Medical

Research, Mill Hill. At Mill Hill, the gel slices were reswollen in a minimal volume of 2ng/μl trypsin (Promega) in 5mM ammonium bicarbonate for in-gel digestion. Peptide mass fingerprinting was performed exactly as described by Field et al (Field et al, 2003).

2.6.4 Small-scale immunoprecipitation and GST-pull down.

Cytoplasmic extracts were incubated either with 1.5μg of antibody and 20μl protein-G/sepharose (Sigma) or with GST-IKKγ truncation mutants pre-bound to glutathione sepharose 4B matrix. The complexes were washed three times in lysis buffer and all liquid was removed. Precipitated proteins were either used in kinase assays (Section 2.5.6) or eluted from the matrix by addition of 30μl of sample buffer, heated to 95°C for 4 min and then stored at -80°C prior to use.

2.6.5 Gel filtration.

Cytoplasmic extracts from $2x10^7$-$2x10^8$ cells were centrifuged at $1x10^5$g for 1h at 4°C. 100μl of the supernatant was loaded on a Superose 6 PC 3.2/30 column (Amersham) previously equilibrated in modified lysis buffer (20mM Tris-HCl pH 7.5, 150mM NaCl, 0.2% NP40, 5% glycerol). The fractionation was performed using an LKB:μseparation unit (Amersham) controlled using Smart Manager 5.1 software. The flow rate of the column was maintained at 40μl.min^{-1} and 22 fractions of 100 μl each were collected. 25μl were separated by acrylamide gel for immunoblotting (Section 2.6.8), whereas 50 μl of each fraction was used for kinase assay (Section 2.6.6). The column was calibrated in modified lysis buffer using protein standards: thyroglobin (669 kDa), ferritin (440 kDa) and catalase (232 kDa) (Amersham).

2.6.6 Kinase assay.

Kinase assays using the anti-vFLIP antibody to isolate IκB kinase activity were performed based on the method of Didonato (DiDonato 2000). 50μl of each column

fraction or 100-200μg of cytoplasmic extract were incubated for 2 h at 4°C with anti-vFLIP antibody and protein-G sepharose. Immune complexes were precipitated and washed three times in 0.5ml of high salt lysis buffer (lysis buffer with 500 mM salt). Immune complexes were washed a further two times in kinase wash buffer before all buffer was removed and 40μl kinase reaction buffer was added. To each reaction, 0.5μl of P^{32}-γ-ATP and 1μg of wildtype IκBα-1-54 or mutant IκBα-1-54-S32A/S36A GST fusion protein was added. The reactions were incubated at 30°C for 30 min and stopped by addition of sample buffer and heated to 95°C for 4 min. The samples were separated by SDS-PAGE and the radiolabelled phosphoproteins were visualised by autoradiography.

For kinase assays using the anti-IKKβ antibody (Santa Cruz-8330), an additional pre-clearance step was included in the protocol. The cytoplasmic extract was incubated for 1h at 4°C with 1.5μg normal rabbit serum and 20μl protein-G sepharose. Immune complexes were precipitated and the soluble fraction was incubated with 1.5μg anti-IKKβ antibody for 2hr. The remaining protocol was performed exactly as described above.

2.6.7 Preparation of nuclear and cytoplasmic fractions.

Cells were washed in cold PBS, pelleted and resuspended in cold NP40 lysis buffer lacking NP40. Following 15 min incubation at 4°C, NP40 was added to a final concentration of 0.6%. The tubes were mixed by vortex and incubated for a further 4 min. The lysate was then underlayered with lysis buffer containing 30% sucrose and centrifuged for 5 min at 4°C. The supernatant was removed as 'cytoplasmic extract', assayed for protein concentration and stored at -80°C. The nuclei were washed twice by overlayering and removing lysis buffer and then resuspended in nuclear lysis buffer. Nuclear proteins were released using 3 freeze-thaw cycles by transferring the tubes from liquid nitrogen to a 37°C water bath. The supernatant, following centrifugation for 10 min at 4°C, was diluted 1:2 in lysis buffer, assayed for protein concentration, and stored at -80°C as 'nuclear extract'.

2.6.8 SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analysis.

Proteins were separated by SDS-PAGE and transferred to hybond ECL nitrocellulose membranes (Amersham) for immunoblot analysis. All blots were incubated overnight at 4°C in blocking solution (PBS containing 2.5% low-fat milk and 0.1% Tween 20) and incubated with primary antibody for 1h in blocking solution. The only exception being the anti-Tax antibody, where 5% BSA was used in place of milk in the blocking solution. Primary antibodies are listed in Table 2.3. Bound antibodies were detected with appropriate peroxidase-conjugated secondary antibodies (1:2000 dilution) and visualised by chemiluminescence reagents (Amersham).

Antigen/Reference	Source	Dilution	Supplier
Haemagglutinin (HA) tag	Rat mAb	1:1000	Roche (867-423)
IκBα	Rabbit pAb	1:200	Santa Cruz (SC-371)
IKKα	Rabbit pAb	1:1000	Cell Signalling Technology (2682)
IKKβ	Goat pAb	1:200	Santa Cruz (SC-7330)
IKKγ	Rabbit pAb	1:200	Santa Cruz (SC-8330)
p100/p52	Rabbit pAb	1:1000	Gift: S. Ley (Mill Hill, UK)
p100/p52	Mouse mAb	1:1000	Upstate Biotech (05-361)
RelB	Rabbit pAb	1:200	Santa Cruz (SC-226)
Sp1	Rabbit pAb	1:200	Santa Cruz (SC-59)
TAT-1 tubulin (Woods et al, 1989)	Mouse mAb	1:1000	Gift: S. Ley (Mill Hill, UK)
Tax (Tanaka et al, 1991)	Mouse mAb	1:1000	Gift: Y.Tanaka (Okinawa, Japan)
VCyclin	Rat mAb	1:100	Gift: S. Mittnacht (Imperial, UK)
vFLIP 6/14 (Low et al, 2001)	Rat mAb	1:100	Gift: W.Low (UCL, UK)
Xpress tag	Mouse mAb	1:5000	Invitrogen (46-0528)

Table 2.3. Primary antibodies.

2.6.9 Electrophoretic mobility shift assay (EMSA).

2.6.9.1 Radioactive labelling of oligonucleotide probes.

NF-κB consensus (SC-2505) and NF-κB mutant (SC-2511) oligonucleotides were purchased from Santa Cruz. T4 polynucleotide kinase (PNK) (Promega) was used to catalyse the transfer of the γ-phosphate group from ATP to the 5'-hydroxyl terminus of each DNA molecule. The constituents of each reaction were:

DNA (10 pmol)	39 µl
Kinase 10x buffer	5 µl
γ-^{32}P-dNTP	5 µl
T4 PNK	1 µl

The reaction was incubated at 37°C for 10 min and then stopped by addition of 2µl 0.5M EDTA. Labelled DNA was purified used Chroma Spin™ columns (Clontech) as per the manufacturer's instructions and stored at -20°C prior to use.

2.6.9.2 EMSA and supershift.

The binding reactions were carried out at 24°C for 15 min. The constituents of each binding reaction were:

2x Parker buffer	10µl
Poly dIdC (Pharmacia) 10ng.µl^{-1}	1 µl
Nuclear extract	5 or 7.5µg
Labelled probe	1 µl
Distilled water	to 20 µl

For supershift analyses, nuclear extracts were pre-incubated with antibodies for 30 min on ice prior to adding the probe. The anti-p50 (06-886) and anti-p52 antibodies (05-361) were purchased from UBI, and the anti-RelB antibody (SC-226 C-19) was purchased from Santa Cruz. A non-denaturing acrylamide gel was pre-run for 1h

before samples were loaded and run at 7.5 volts.cm^{-1} at 24°C. Bandshifts were visualised by autoradiography.

2.6.10 Luciferase based reporter assays.

To assay NF-κB activity, 293T cells in 24-well plates were transiently co-transfected with 80ng of pGL2 promoter vector and either vFLIP or Tax, or stimulated with TNFα as stated. At 48h post-transfection, cells were washed in PBS and lysed in reporter lysis buffer (Promega). Luciferase activities were measured by luminometer in cleared lysates with the luciferase assay system (Promega) as per the manufacturer's instructions. NF-κB activity was calculated as fold induction compared to that of empty vector transfected controls.

CHAPTER 3

vFLIP binds and activates IKK in PELs

3.1 Introduction

Two roles have been proposed for vFLIP in KSHV infection: inhibition of DR-mediated apoptosis and activation of NF-κB (Section 1.4.7). Concurrent with the work described in this thesis, Liu et al described the interaction between vFLIP and the IKK complex and the activation of the IKK complex when vFLIP was ectopically expressed in a non-small-cell lung carcinoma cell line (Liu et al, 2002). This chapter focuses on the functions and biochemical properties of endogenous vFLIP in the PEL cell line, BC3 (Section 2.4.1).

3.2 Mass spectrometry identification of proteins interacting with vFLIP in BC3 cells

vFLIP was purified from BC3 cells by immunoprecipitation using the monoclonal anti-vFLIP antibody 6/14 (Low et al, 2001). $5x10^9$ cells were lysed in 0.2% NP40 detergent to release cytoplasmic proteins but minimise disruption of protein interactions. 1mg of antibody coupled to sepharose beads was sufficient to deplete vFLIP from this lysate (data not shown). The immune complexes were washed in high salt buffer (0.5M NaCl), and proteins that co-immunoprecipitated with vFLIP but were not precipitated by a control rat antibody were excised and identified by mass spectrometry. Fig. 3.1 shows that five proteins were identified in the vFLIP lane but not in the control lane. All five proteins were clear matches with high Mascot scores (Perkins et al, 1999). Three of these proteins were identified as the core components (IKKα, IKKβ and IKKγ) of the IKK complex. The band containing IKKα was also found to contain the chaperone protein, Hsp90, which has recently been identified as an additional component of the IKK complex (Section 1.3.3.2). The fifth protein was identified as vFLIP. Since vFLIP was recovered from the immunoprecipitation at similar levels to the IKK components, it is unlikely that these proteins were contaminants.

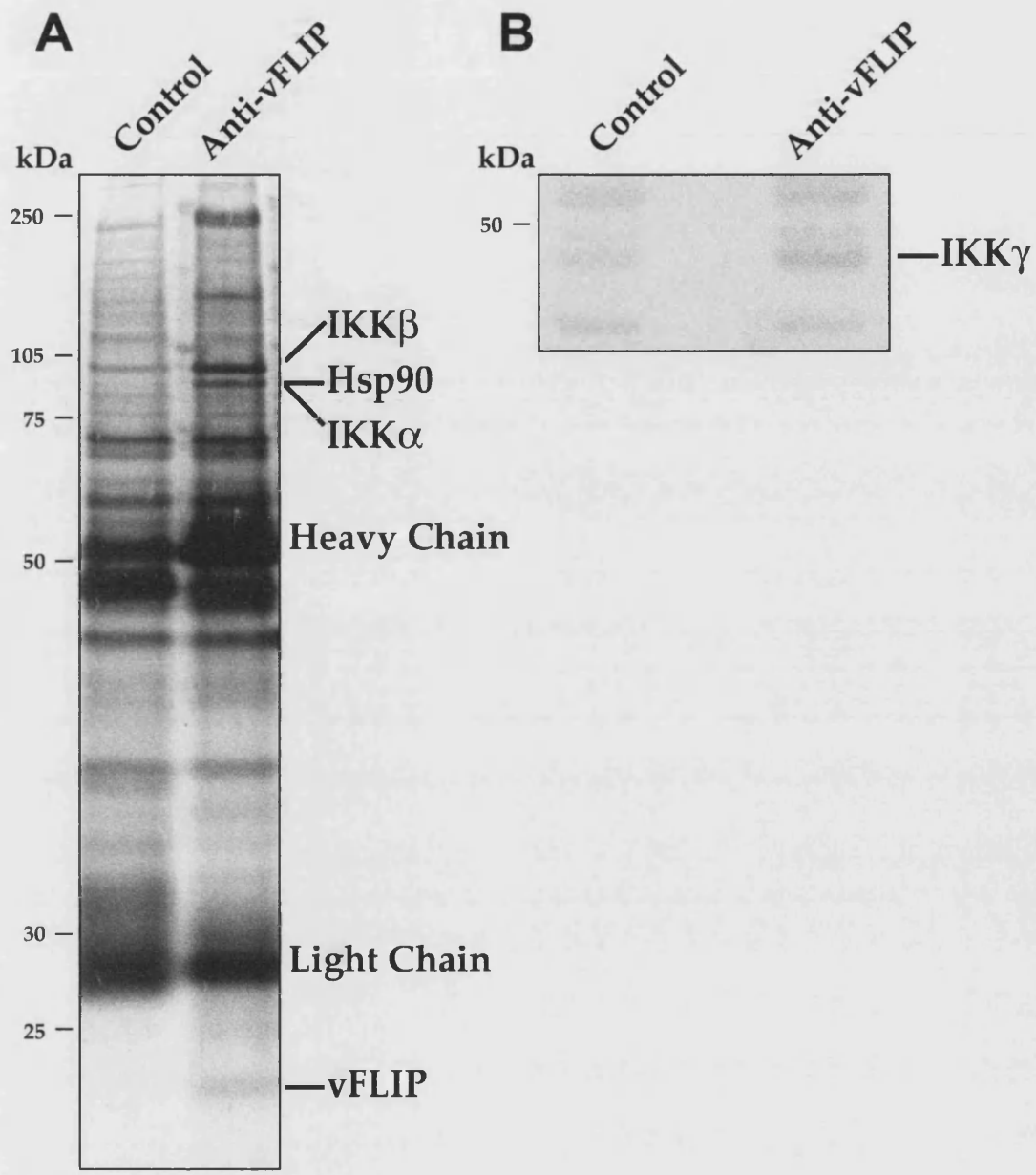


Figure 3.1. Identification of proteins interacting with vFLIP in a KSHV infected cell line.

Cytoplasmic extract (0.2% NP40 buffer) from the KSHV-infected PEL cell line BC3 ($5x10^9$ cells per track) was immunoprecipitated using a rat IgG control or the anti-vFLIP monoclonal 6/14 antibody, and then run on a 12% SDS-polyacrylamide gel. (A) Silver stain of tracks containing 10% of the immunoprecipitated protein. (B) Colloidal Coomassie stain of part of a parallel pair of tracks containing 90% of the immunoprecipitated protein. Bands detected in the vFLIP immunoprecipitate, but not the control, were excised from the Coomassie stained gel and identified by mass spectrometry as indicated.

3.3 Analysis of the endogenous vFLIP-IKK complex

Biochemical analysis has defined the IKK complex as a 700-900 kDa multi-protein complex (Section 1.3.3.2). Gel filtration analysis was therefore used to examine the distribution of vFLIP protein in BC3 cells. The cell extract was eluted into 22 fractions using a Superose 6 column, and analysed by immunoblot. Fig. 3.2 demonstrates the presence of vFLIP in a high molecular weight protein complex at a peak corresponding to an exclusion size of 700 kDa. The three components of the IKK complex (IKKα, IKKβ and IKKγ) were co-eluted with vFLIP. Although vFLIP has a predicted molecular weight of 23 kDa, vFLIP was not detected in any smaller molecular weight fractions. The KSHV vcyclin protein is of similar molecular weight to vFLIP (predicted 28 kDa) and was observed in fractions 11 and 12, consistent with a molecular weight of less than 150 kDa.

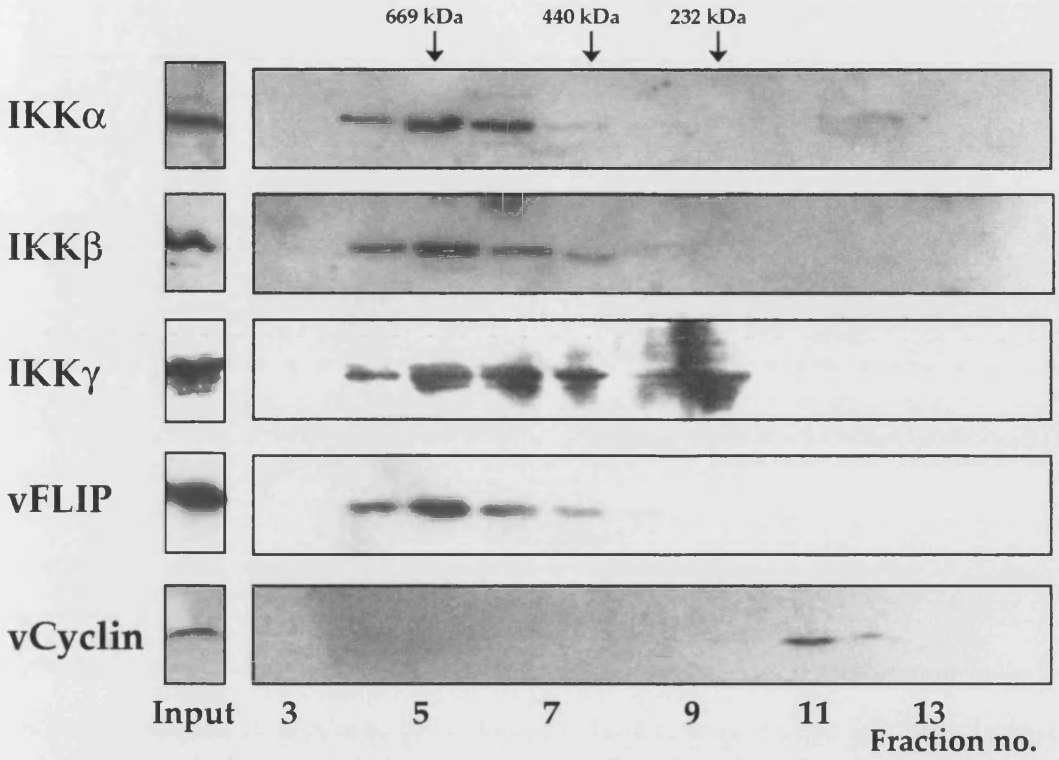


Figure 3.2. Analysis of the vFLIP-IKK complex by gel filtration.

Cytoplasmic extract (0.2% NP40 buffer) from BC3 cells ($2x10^8$) was subjected to gel filtration on a Superose 6 column. Fractions were then analysed by immunoblot using anti-IKKα, β and γ, anti-vFLIP and anti-vCyclin antibodies. The elution volume of protein standards is indicated.

Gel filtration experiments therefore suggest a single population of soluble vFLIP in BC3 cells. These data imply that the main function of vFLIP in these cells is to bind IKK. However, it remained possible that a proportion of the vFLIP in this high molecular weight complex was not IKK associated. To address this question, the IKKγ immunoprecipitate from BC3 cells was analysed by immunoblot to determine the proportion of IKKγ-associated vFLIP. Fig. 3.3.A demonstrates that all the detectable vFLIP in BC3 cell lysate was associated with IKKγ, since immunoprecipitation using the anti-IKKγ antibody could completely deplete vFLIP from the cell lysate, but did not affect IκBα.

The methodology for the identification of IKK components in complex with vFLIP (Fig. 3.1) included three washes of the immune complexes in 0.5M NaCl (see Materials and Methods). To further assess the stability of the vFLIP-IKK complex, equal proportions of anti-vFLIP immunoprecipitate were subjected to three washes with either the lysis buffer alone, the buffer containing 1% Triton X-100 or the buffer containing 2M urea (Fig. 3.3.B). Immunoblotting analyses show that the association of vFLIP with IKK is resistant to both detergent and denaturing washes. These results demonstrate the stability of the vFLIP-IKK complex in high salt, detergent and denaturing conditions, implying that the association between vFLIP and the IKK complex is strong.

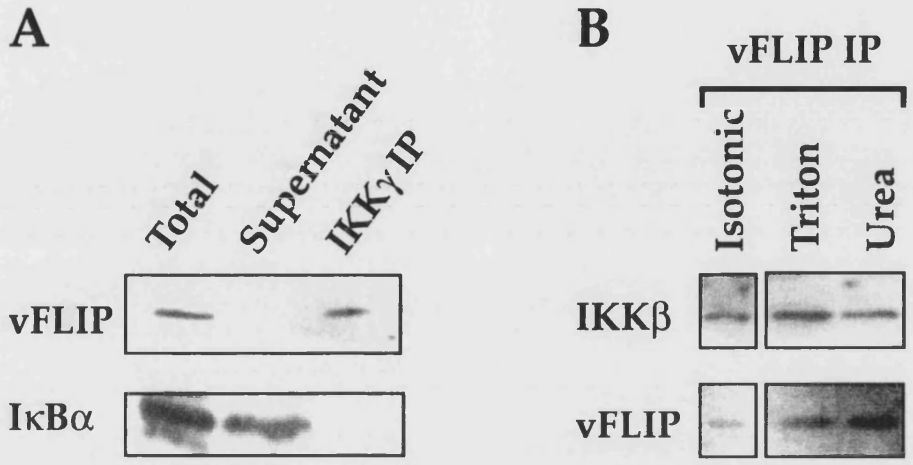


Figure 3.3. Further analysis of vFLIP-IKK.

(A) Cytoplasmic extract (0.2% NP40 buffer) from $5x10^6$ BC3 cells was immunoprecipitated using an anti-IKKγ antibody. The immunoprecipitate (IKKγ IP) and 5% of both the original extract (Total) and the supernatant from the immunoprecipitation (Supernatant) were then analysed by immunoblot with anti-vFLIP and anti-IκBα antibodies.
(B) Cytoplasmic extract (0.2% NP40 buffer) from $1x10^7$ BC3 cells was immunoprecipitated using the anti-vFLIP antibody. Equal portions of immune complex were washed three times in lysis buffer alone (Isotonic) or the buffer containing either 1% Triton X-100 (Triton) or 2.0M urea (Urea) and analysed by immunoblot with anti-IKKβ and anti-vFLIP antibodies.

3.4 vFLIP has associated kinase activity in BC3 cells

Superose 6 fractions were also analysed for IκBα kinase activity associated with vFLIP (Fig. 3.4.A). Fractions were immunoprecipitated using the anti-vFLIP antibody and immune complexes were subjected to *in vitro* kinase assay. The substrate for this reaction was an N-terminal IκBα fragment (amino acids 1–54) fused to GST (Sections 2.3.2; 2.5), since full-length GST-IκBα was phosphorylated non-specifically (data not shown), probably due to phosphorylation sites in the C-terminal portion of the protein (DiDonato et al, 1997; Zandi et al, 1997). Kinase activity was found to be associated with vFLIP in fractions 4-7, with the major peak in fraction 5, identical to the distribution by immunoblot of vFLIP and IKK in Fig. 3.2. Fig. 3.4.B demonstrates the specificity of this kinase assay. Immune complexes precipitated using an isotype-matched control rat antibody did not have an associated kinase activity, and the vFLIP immune complex was not able to phosphorylate GST-IκBα-S32A/S36A, a mutant containing point mutations at the inducible phosphorylation sites (Sections 2.3.2; 2.5).

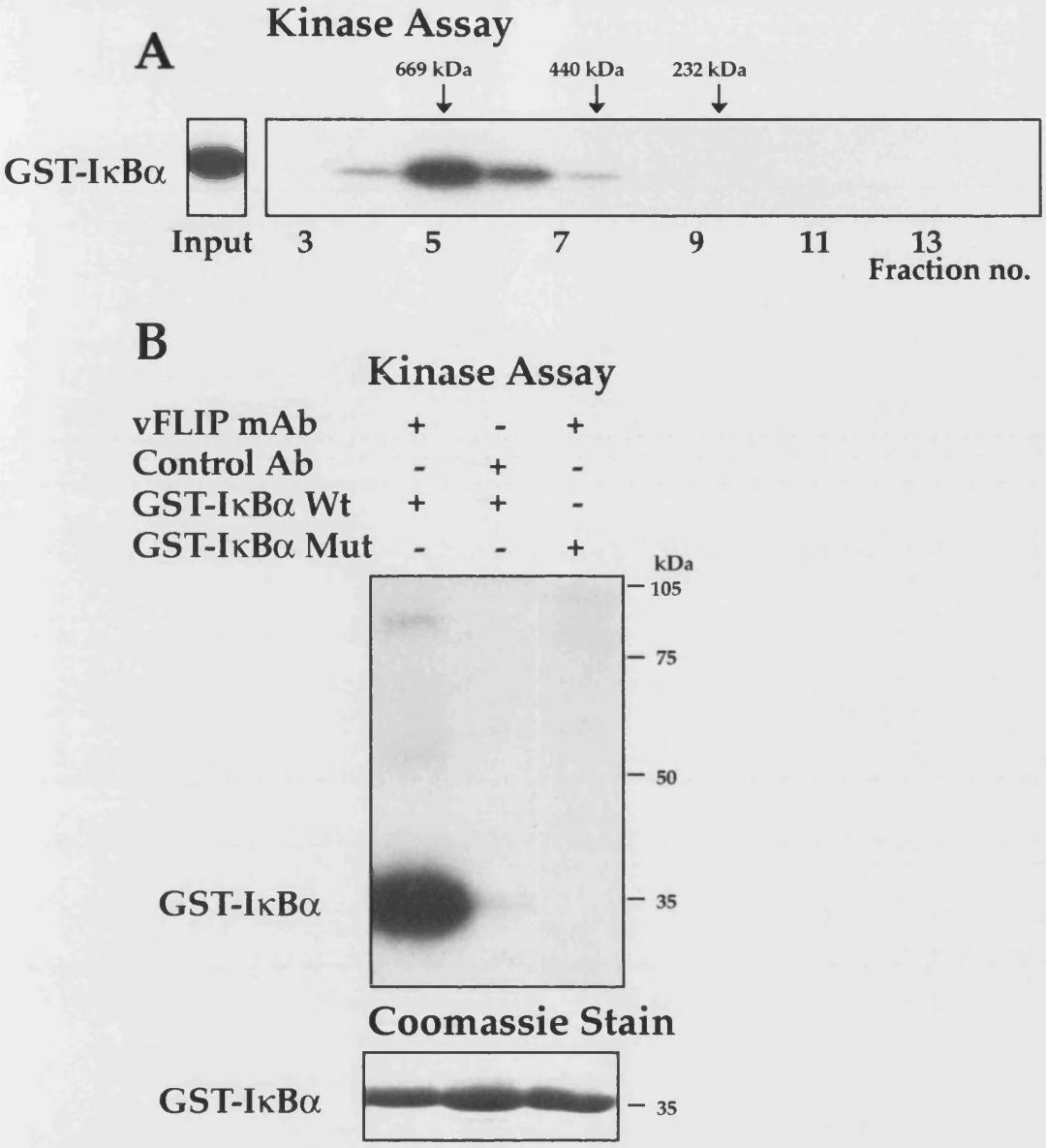


Figure 3.4. vFLIP has associated kinase activity.

(A) Superose 6 fractions from BC3 cells, as described in Figure 3.2, were immunoprecipitated with the anti-vFLIP antibody and the immune complexes were incubated for 0.5h with GST-IκBα and γ–P^{32}-ATP. Radiolabelled proteins were resolved on an SDS-polyacrylamide gel and visualised by autoradiography.
(B) Cytoplasmic extracts from BC3 cells were immuno-precipitated using the anti-vFLIP antibody or an isotype-matched control. The immune complexes were incubated for 0.5h with wildtype or mutant (S32A/S36A) GST-IκBα substrates and γ–P^{32}-ATP. Radiolabelled proteins were resolved on an SDS-polyacrylamide gel and visualised by autoradiography (upper panel). The lower panel shows a Coomassie stained gel of the GST-IκBα in each reaction.

3.5 vFLIP in heterologous cells

In the context of KSHV infection, other viral proteins might cooperate with vFLIP to activate IKK (Table 1.2). The DEL cell line (Section 2.4.1), a KSHV negative and EBV negative B cell line, was chosen to investigate whether vFLIP alone was sufficient to activate the IKK complex. DEL cells were transduced with a lentiviral vector expressing both vFLIP fused to an N-terminal HA epitope tag (HA-vFLIP) and GFP. However, Fig. 3.5.A shows that HA-vFLIP did not co-elute with IKK in transduced DEL cells when lysates were subjected to gel filtration (cf. Fig. 3.2). To examine whether the failure of HA-vFLIP to bind IKK was cell specific or due to the HA epitope tag, 293T cells (Section 2.4.1) were transduced with lentivirus encoding either GFP, HA-vFLIP and GFP, or wild-type vFLIP and GFP, and were compared to BC3 cells. Fig. 3.5.B shows that IKKβ and IKK activity were associated with wild-type vFLIP in both BC3 and the transduced 293T cells. However, HA-vFLIP was not able to immunoprecipitate IKKβ or an associated kinase activity. It seems likely that the HA epitope induces a conformational change in vFLIP that prevents its physical interaction with IKK.

293T cells provide an efficient and accessible system in which to model vFLIP activation of IKK. They possess no detectable IKK kinase activity or NF-κB transcriptional activity in the absence of exogenous stimulation, such as TNF. Since the level of expression of proteins containing DED domains can affect their behaviour (Boldin et al, 1996; Han et al, 1997; Shu et al, 1997; Chang et al, 2002), 293T cells were used to investigate the level of expression that is required for vFLIP to activate the IKK complex. 293T cells were infected with a range of MOI from 0.1 to 5 infectious units per cell of lentivirus expressing vFLIP and GFP or GFP alone. vFLIP expression and its associated kinase activity in these cells was compared to that of endogenous vFLIP from the same number of BC3 cells (Fig. 3.6.A). As an example, the cells in Lane 6 express similar levels of vFLIP to the BC3 cells. FACScan analysis indicates 25% efficiency of transduction in these cells. The level of vFLIP expression in these cells is therefore not more than four-fold greater than BC3. The implication is that vFLIP can activate IKK independently and that the degree of activation is not

dissimilar to that found in BC3 cells. These data are consistent with concurrent results published by Chaudhary and colleagues (Liu et al, 2002).

TNFα is a potent but transient activator of IKK in 293T cells (DiDonato et al, 1997; Zandi et al, 1997). TNFα was used to stimulate IKK in 293T cells infected with lentivirus encoding either vFLIP and GFP or GFP alone. Kinase activity was measured using an anti-IKKβ antibody to purify the IKK complex. Fig. 3.6.B shows that 293T cells expressing vFLIP at two weeks post-infection contain a higher basal level of kinase activity than could be achieved using TNFα. These cells were cultured with no obvious change in the level of kinase activity associated with vFLIP. It is concluded that vFLIP activation of IKK is both potent, and is resistant to the normal feedback mechanisms which rapidly down-regulate IKK activation following physiological stimulation.

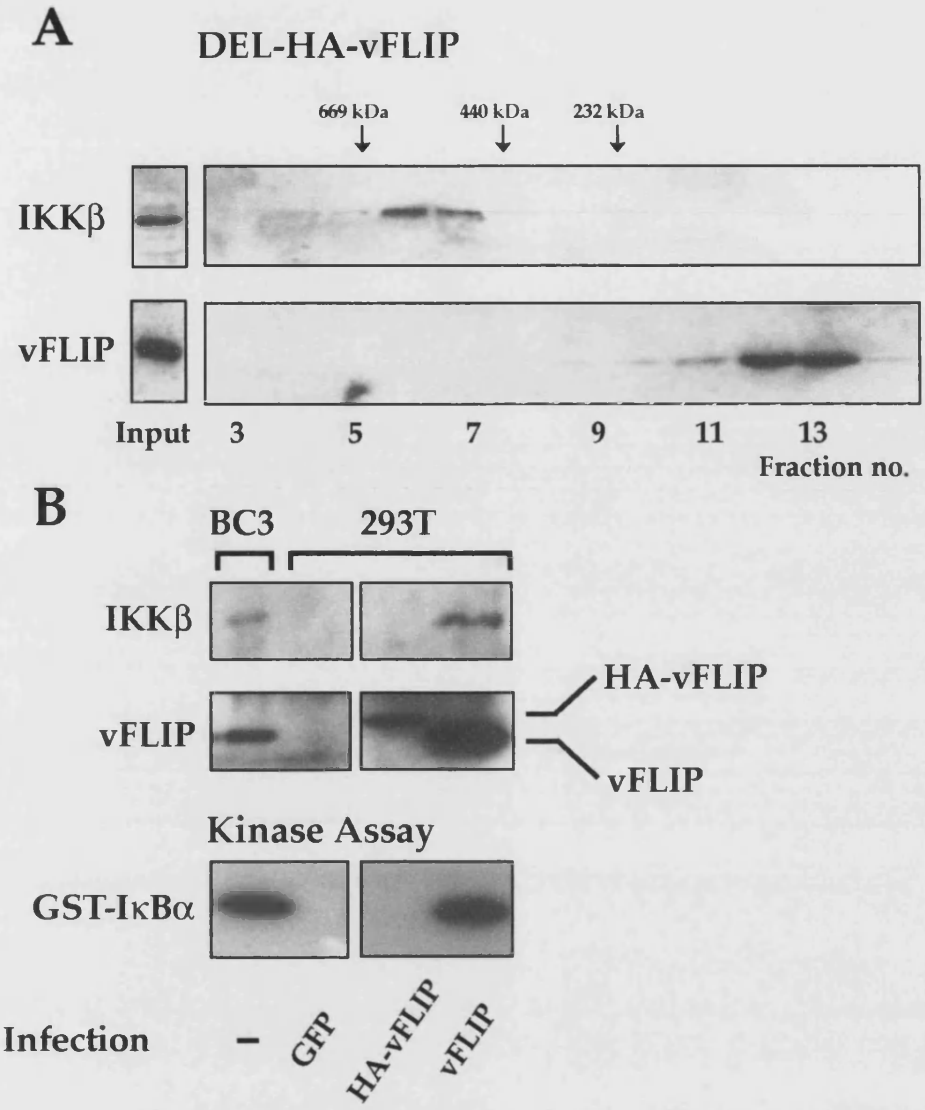


Figure 3.5. Analysis of N-terminal HA tagged vFLIP.

(A) The KSHV negative PEL cell line, DEL, was transduced with lentivirus encoding GFP and vFLIP fused to an N-terminal HA eptitope tag (HA-vFLIP). Cytoplasmic extract (0.2% NP40 buffer) from $2x10^8$ cells was subjected to gel filtration on a Superose 6 column. Fractions were then analysed by immunoblot using anti-IKKβ and anti-vFLIP antibodies.
(B) Cell lysates (0.2% NP40 buffer) from $1x10^7$ BC3 cells or 293T cells infected with lentivirus encoding either GFP alone, HA-vFLIP plus GFP or wildtype vFLIP plus GFP were immunoprecipitated with the vFLIP antibody. 50% of the immune complexes from each cell line was subjected to immunoblot using antibodies to detect vFLIP and co-purified IKKβ. The remaining 50% was assayed for IκBα kinase activity (lower panel).

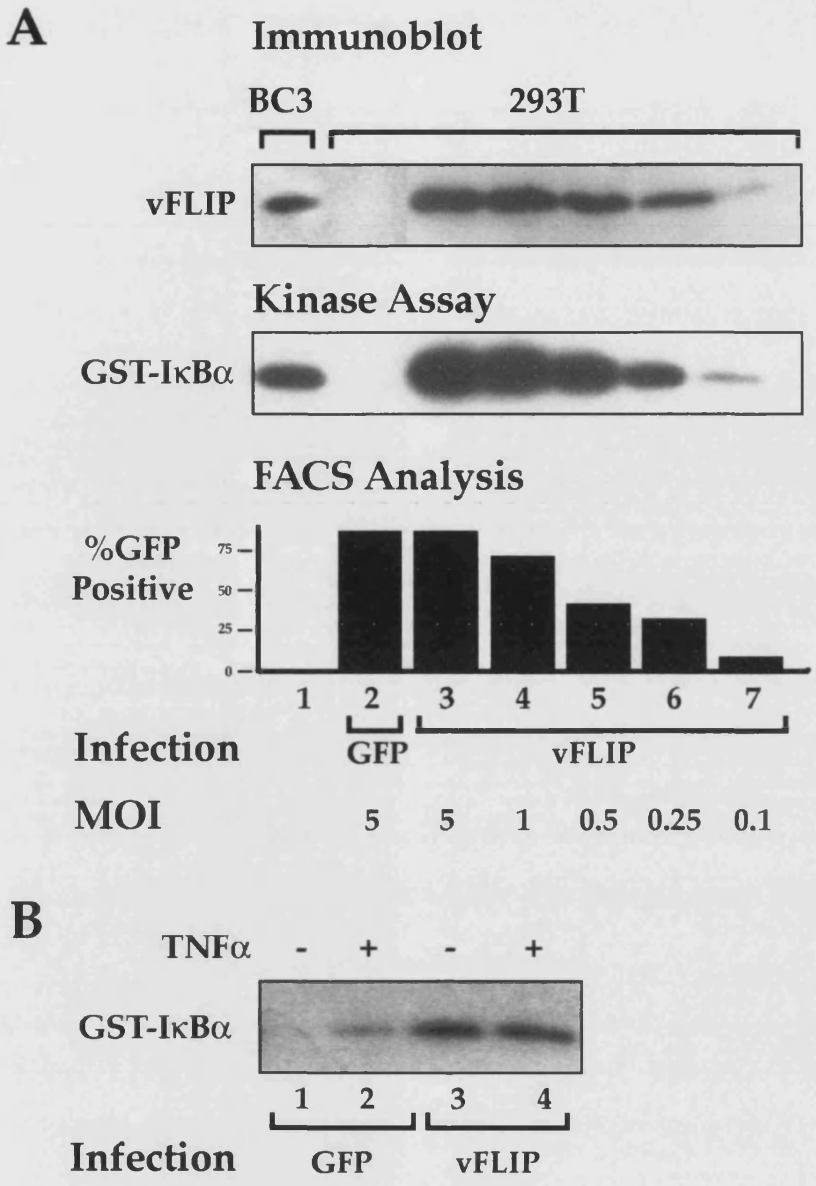


Figure 3.6. vFLIP activation of IKK is independent of KSHV- or B cell associated co-factors.

(A) Cell lysates (0.2% NP40 buffer) from $1x10^7$ BC3 cells or 293T cells infected with lentivirus encoding either GFP or vFLIP plus GFP at a range of MOI were immunoprecipitated using the anti-vFLIP antibody. The multiplicity of infection (M.O.I) is indictated below. 50% of the immune complexes from each cell line was analysed by immunoblot using the anti-vFLIP antibody (top panel), and the remaining 50% was assayed for IκBα kinase activity (central panel). Transduction efficiency was measured by FACScan analysis of cells expressing GFP (lower panel).
(B) $5x10^6$ 293T cells infected with lentivirus encoding either GFP, or vFLIP plus GFP at two weeks post-infection were either treated or not with 10ng/ml TNFα for 10 min. Cell lysates (0.2% NP40 buffer) were immunoprecipitated with the anti-IKKβ antibody and assayed for IκBα kinase activity.

3.6 Activity of the vFLIP-IKK complex depends on Hsp90

Hsp90 has been previously shown to interact with the kinase domains of IKKα and IKKβ (Chen et al, 2002). The benzoquinone ansamycin anti-tumour agent, geldanamycin (GA), interacts with Hsp90 to inhibit its normal functions (Whitesell et al, 1994). Chen et al, demonstrated that GA treatment disrupted the IKK complex leading to the dissociation of IKKγ and prevented TNF-induced IKK activation (Chen et al, 2002). GA was therefore used to investigate the role of Hsp90 in the vFLIP-IKK complex in 293T cells. The cells were treated with 0.5μM GA in serum free medium for 16h. Gel filtration showed that there was no change in the size of the vFLIP complex upon treatment with GA (Fig. 3.7.A, B). There was also no change in the levels of IKKα, IKKβ (data not shown) or IKKγ (Fig. 3.7.A, B) expression, and there was no significant change in the dissociation of IKKγ from the complex in control cells expressing GFP alone (Fig. 3.7.C). Although vFLIP was observed in lower fractions in both control and GA-treated cells, this finding was attributed to vFLIP being expressed in excess of the IKK components. However, the IKK activity associated with vFLIP in cells treated with GA was significantly reduced (Fig. 3.7.B). The activity of the vFLIP-IKK complex is therefore dependent on Hsp90.

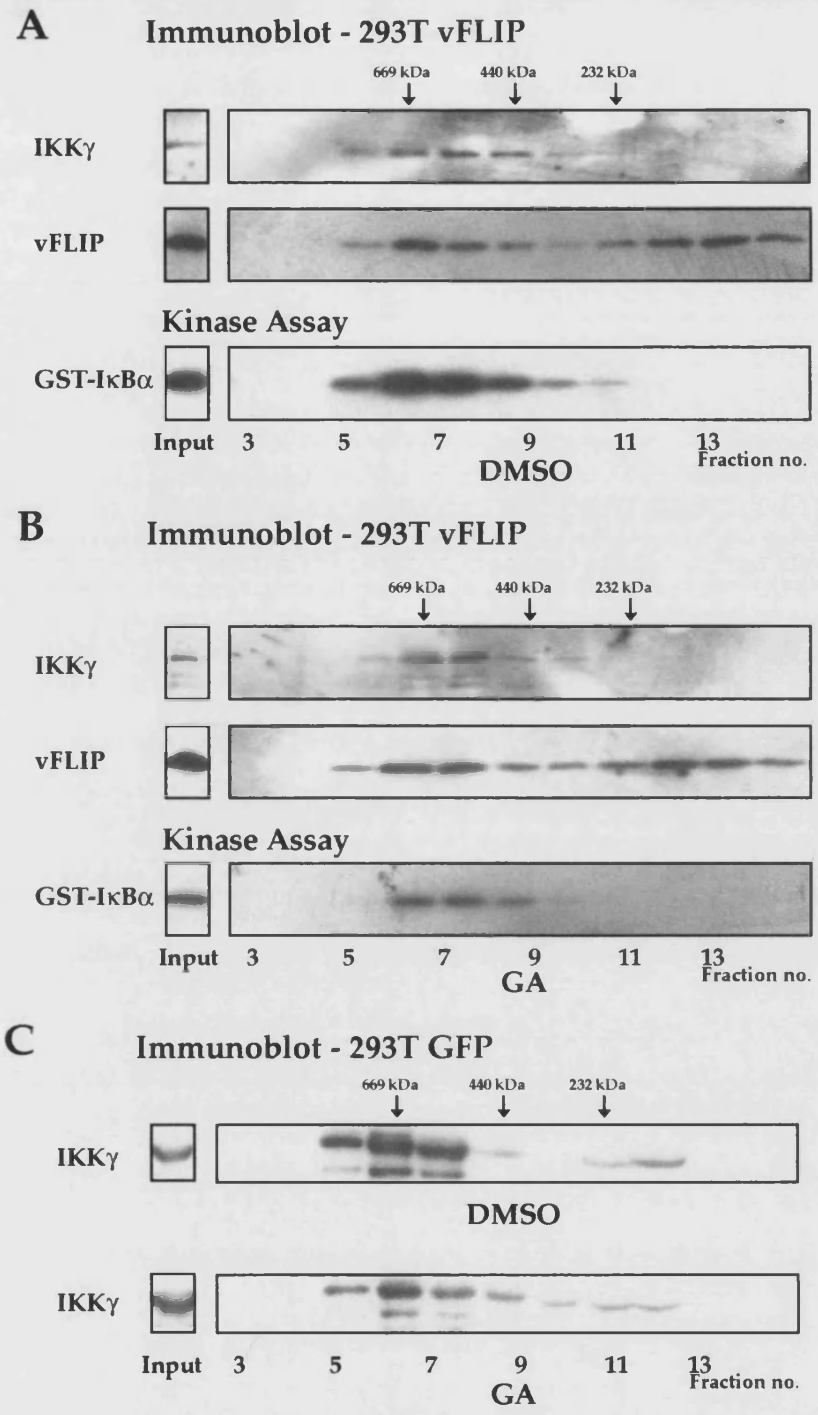


Figure 3.7. The vFLIP-IKK kinase activity is inhibited by geldanamycin.

Cytoplasmic extract (0.2% NP40 buffer) from $1x10^8$ 293T cells transduced with a lentivirus encoding either vFLIP plus GFP (A,B) or GFP alone (C) and then treated with DMSO or 0.5μM geldanamycin (GA) for 15h was subjected to gel filtration on a Superose 6 column. Fractions were analysed by immunoblot using anti-IKKγ or anti-vFLIP antibodies; in A and B fractions were also immunoprecipitated with the anti-vFLIP antibody and immune complexes were assayed for IκBα kinase activity.

3.7 GA effect on IKK and survival in BC3 cells

Constitutive activation of NF-κB is associated with KSHV infection in PEL cells (Keller et al, 2000; Sun et al, 2003a). These cells undergo apoptosis when challenged with the inhibitor of cytokine-inducible IκBα phoshorylation, Bay 11-7082 (Keller et al, 2000). To evaluate the effect of GA on KSVH-infected cells, BC3 were treated for 16h with 0.5 μM GA and vFLIP-associated kinase activity was measured. Fig. 3.8.A shows that GA significantly inhibited the kinase activity associated with vFLIP compared to control cells. At 48h, this concentration of GA also caused loss of viability of BC3 cells (Fig. 3.8.B). As an example, 72% of GA-treated cells were dead at 48h, compared with 35% of control cells treated with DMSO in serum-free medium. To investigate the mechanism of cell death, cells were stained with Annexin V and propidium iodide. Early stage apoptosis is distinguished by the translocation of phosphatidyl serine (PS) from the cytosolic to the outer surface of the cytoplasmic membrane (Fadok et al, 1992). Annexin V binds PS (Zhang et al, 1997) and therefore identifies early apoptotic cells (Koopman et al, 1994; Zhang et al, 1997; van Engeland et al, 1998). Annexin V also binds necrotic cells, but these cells are permeable to propidium iodide (PI). Since early apoptotic cells are impermeable to PI, use of annexin V in conjunction with PI allows for differentiation between early apoptotic cells (annexin V positive), late apoptotic or necrotic cells (PI and annexin V positive) and viable cells (unstained) (Vermes et al, 1995). Treatment with GA caused a significant increase in double positive cells (Fig. 3.9). This result does not clearly define the mechanism of cell death in these cells.

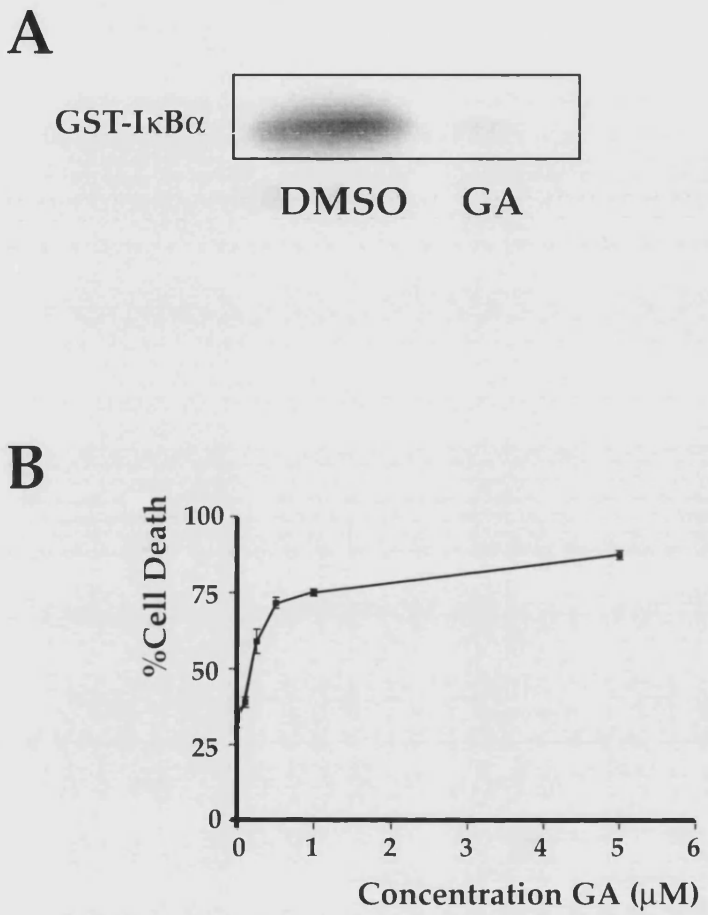


Figure 3.8. BC3 cells are killed by geldanamycin.

(A) Cytoplasmic extracts from $5x10^6$ BC3 cells treated with DMSO or 0.5μM GA for 15h were immunoprecipitated using the anti-vFLIP antibody and the IκBα kinase activity of the immune complexes was measured.
(B) The viability of BC3 cells treated with increasing concentrations of GA for 48h was measured using a haemocytometer to count the number of live and dead cells and calculate the percentage of dead cells. Standard error of the mean (SEM) error bars are calculated on the basis of triplicate counts.

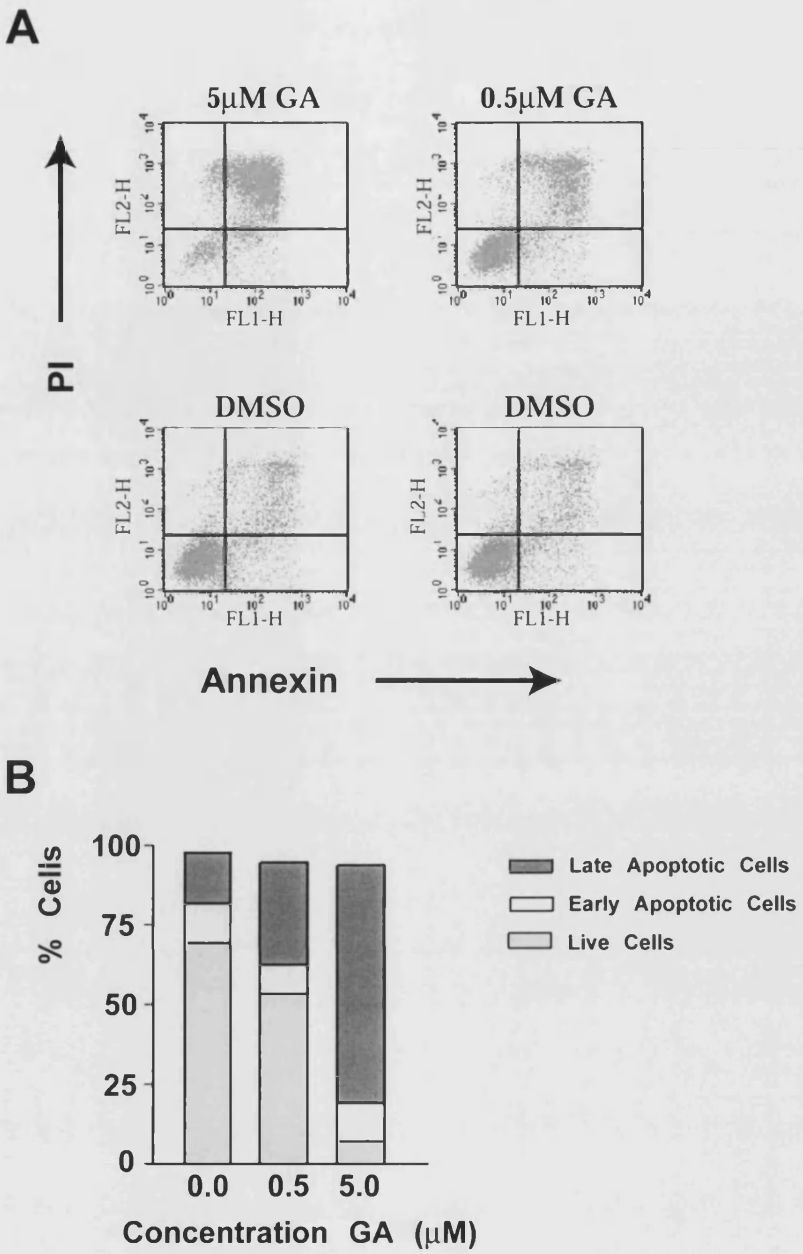


Figure 3.9. Cell death in BC3 cells treated with geldanamycin evaluated by propidium iodide/annexin V staining.

(A) The viability of BC3 cells treated with two concentrations of GA or equivalent DMSO at 48h was measured by propidium iodide (PI)/ annexin V stain followed by FACScan analysis. In each graph, the lower left quadrant represents live unstained cells, the lower right quadrant represents single stained early apoptotic cells, and the upper right represents double stained late apoptotic cells. These data are reproduced in bar chart format (B).

3.8 Discussion

This chapter focuses on the physical interactions and biochemical properties of KSHV vFLIP in latently infected PEL cells. Chaudhary and colleagues, have recently demonstrated that vFLIP associates with and activates IKK when expressed in human non-small cell lung carcinoma cells (Liu et al, 2002). However, by analogy with other FLIP proteins, KSHV vFLIP is anticipated to inhibit pro-caspase-8 maturation. The data presented in this thesis support the observations made by Chaudhary et al, and extends their conclusions to a KSHV-infected PEL cell line. Using immunoprecipitation and gel filtration, vFLIP is demonstrated to associate with an activated IKK complex in BC3 cells. At the level of detection by immunoblot, the majority of vFLIP exists as a highly stable complex with IKK. This implies that activation of IKK is the primary role for vFLIP in latently infected PEL cells.

A number of candidate KSHV genes have been put forward to explain the constitutive NF-κB activation observed in KSHV infection of B cells (Section 1.4.7; Table 1.2). The observation that vFLIP-IKK is constitutively active in BC3 cells lends considerable weight to the argument that vFLIP is responsible. This argument is further supported by observations that vFLIP binds and activates IKK in 293T cells (Fig. 3.5) and other non-lymphoid cells (Liu et al, 2002). The lentivirus system enabled the stable expression of vFLIP at near-physiological levels in 293T cells, and the level of kinase activity measured *in vitro* was similar to that found in BC3 cells. It is concluded that vFLIP is able to activate the NF-κB pathway independently of KSHV- and B cell associated co-factors with a potency that may be sufficient to account for the level of NF-κB activation that is observed in KSHV-infected PEL cells.

In most cell types, IKK activity is found to peak at 5-15 min. At 30 min, activity is reduced by 75% and it decreases further over the next 90 min (DiDonato et al, 1997; Zandi et al, 1997; Delhase et al, 1999). The initial down-regulation of IKK is due to phosphorylation of residues in the C-terminus of IKKα and IKKβ (Delhase et al, 1999). Since substitution of 10 serine residues in the C-terminus of IKKβ for alanine

led to a four-fold increase in the length of time that IKK activity was sustained, while glutamic acid substitution for the same residues significantly inhibited TNFα-induced activity (Delhase et al, 1999). In contrast to the transient activation of IKK by TNFα, the level of vFLIP-induced kinase activity in 293T cells remained constant over a period of weeks in culture. This indicates that the vFLIP-IKK complex is somehow refractory to the normal feedback mechanisms that regulate IKK activity.

In addition to the IKK subunits, we found Hsp90 to be 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 study demonstrated a physiological role for Hsp90 and Cdc37 in TNFα-dependent activation of IKK, showing that GA prevented membrane recruitment and activation of IKK upon TNFα treatment. In another study, GA induced the degradation of RIP (Lewis et al, 2000), a kinase essential for TNF-induced NF-κB activation (Kelliher et al, 1998). GA also inhibited the activity of the vFLIP-IKK complex, although the dissociation of IKKγ from the complex reported by Chen et al, was not observed (Chen et al, 2002). These data demonstrate a second mode of IKK activation that can be inhibited by GA, and support a role for Hsp90 in the IKK complex. Consistent with the inhibition of NF-κB in PEL cells (Keller et al, 2000), GA also induced death in BC3 cells. This implies that vFLIP activation of IKK is essential for the survival of BC3 cells. Indeed, Sun et al, have shown that vFLIP activation of NF-κB protects against growth factor withdrawal-induced apoptosis in a leukaemic cell line (Sun et al, 2003a). However, a recent publication by Brown et al (Brown et al, 2003), suggests an alternative explanation. This study demonstrates that NF-κB inhibits the activation of lytic promoters in three herpesviruses: KSHV, EBV and MHV68. Upon treatment with Bay11-7082 (section 1.4.7), these viruses initiate lytic protein synthesis (Brown et al, 2003). It is therefore possible that lytic reactivation of KSHV is responsible for the cell death seen in these experiments.

CHAPTER 4

Characterising the association between vFLIP and IKK.

4.1 Introduction

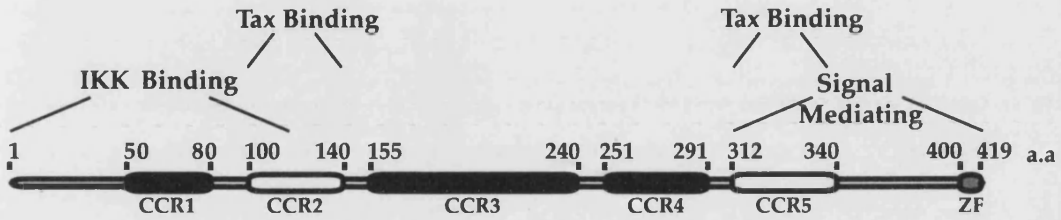
4.1.1 Overview

Since the core components of IKK exist as a large and stable complex in mammalian cells, it was impossible to predict which component was required by vFLIP to contact IKK. This chapter describes the direct physical association between vFLIP and the IKKγ subunit of IKK.

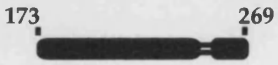
4.1.2 The IKKγ subunit

IKKγ/NEMO was discovered in a genetic complementation assay as a cellular factor that restored NF-κB activation in cells resistant to normal stimulation of the pathway (Yamaoka et al, 1998). IKKγ was also isolated independently as a component of the high molecular weight IKK complex (Rothwarf et al, 1998; Mercurio et al, 1999), and as a factor binding to adenovirus E3-14.7K protein (Li et al, 1999c). It is a highly conserved glutamine-rich protein, and in humans contains 419 amino acids. Structural predictions indicate a C-terminal zinc finger, and five extended coiled-coil regions, of which the second and fifth from the N-terminus contain leucine zipper motifs (LZ1 and LZ2, respectively), predicted according to Lupas et al (Lupas et al, 1991; Chu et al, 1999) (Fig. 4.1.A). The role of IKKγ is described in more detail in Section 1.3.3.2.

A IKK gamma



B Putative minimum binding domain



C Yeast-two-hybrid Screen

Fusion #	Coding Start (aa)	Coding Stop (aa)	Frequency
1	1	269	4
2	4	389	2
3	36	371	2
4	37	286	4
5	150	372	1
6	173	390	1

Figure 4.1. Yeast-two-hybrid data suggest a direct interaction between vFLIP and IKKγ.

A human placental cDNA library was screened for proteins interacting with a vFLIP bait using yeast-two-hybrid technology by our collaborators at Hybrigenics. (A) A schematic representation of IKKγ with the putative minumum domain required for the interaction (B) and a list of the six independent fusions of IKKγ that were identified (C). Black boxes indicate coiled-coil regions (CCR), white boxes indicate the CCRs containing leucine zipper motifs essential for the interaction of HTLV-1 Tax with IKKγ and the grey box indicates a zinc finger motif (ZF).

4.1.3 Genetic analysis of IKKγ

Single copy deletion of IKKγ in mice results in the death of male mice and an inflammatory condition in females characterised by granulocyte infiltration of the skin, with both hyperproliferation and increased apoptosis of karatinocytes (Makris et al, 2000; Schmidt-Supprian et al, 2000). Homozygous deletion of IKKγ is lethal at E12.5-13.0 in female mice. IKKγ is the only component of the IKK complex linked to human disease, mutations within the IKKγ gene being associated with two X-linked human disorders. Familial incontinentia pigmenti (IP) causes granulocyte infiltration of the skin in females and male prenatal lethality. An identical genomic deletion within IKKγ, that causes protein truncation and eliminates IKK activity, accounts for 90% of all cases of IP (Smahi et al, 2000; Aradhya et al, 2001). Hypohidrotic ectodermal dysplasia with immune deficiency (HED-ID) is X-linked recessive, and normally benign in comparison to IP. HED-ID is often associated with mutations in the zinc finger of IKKγ (Zonana et al, 2000; Jain et al, 2001; Doffinger et al, 2001). These mutations impair, but do not generally abolish NF-κB activation, and this may explain why HED-ID is less severe than IP. These genetic studies highlight the critical role of IKKγ in regulating the IKK complex and NF-κB pathway.

4.1.4 IKKγ interacting proteins.

At least six proteins are known to interact with IKKγ. Receptor interacting protein (RIP) is an adaptor protein that associates with TNF-receptor 1 (TNFR1) and is required for NF-κB activation via this receptor (Ting et al, 1996; Kelliher et al, 1998). RIP contains three domains: an N-terminal kinase domain, an intermediate domain, and a C-terminal death domain. RIP interacts with IKKγ (Li et al, 1999c; Zhang et al, 2000; Ye et al, 2000) via its intermediate domain (Zhang et al, 2000), and it has been proposed that RIP stimulates the oligomerisation of IKKγ to activate IKK (Poyet et al, 2000). Another protein, A20, can inhibit the TNFR1 signalling pathway leading to apoptosis (He et al, 2002), and has been reported to interact with IKKγ (Zhang et al, 2000). However, He et al, demonstrate normal anti-apoptotic activity of A20 in a

IKKγ-deficient Jurkat cell line, inferring that the IKKγ-A20 interaction is not required for this activity (He et al, 2002). Most recently, the familial cylindromatosis tumour suppressor, CYLD, has been shown to interact with IKKγ (Section 1.3.3.4). Three independent studies reported that CYLD functions as a de-ubiquitinating (DUB) enzyme with specificity towards TRAF2. Bridging an interaction between IKKγ and TRAF2, CYLD was shown to negatively regulate IKK activation by TRAF2 and various TNF receptors to inhibit NF-κB. Intriguingly, loss of DUB activity through CYLP truncation is associated with oncogenesis (Brummelkamp et al, 2003; Trompouki et al, 2003; Kovalenko et al, 2003).

IKKγ is also the point of access to the NF-κB pathway for three viral proteins. It was cloned as a factor, designated FIP-3, that binds to adenovirus protein Ad E3-14.7K and prevents the cytolytic effects of TNFα (Li et al, 1999c). Since the TNFα-mediated immune response and NF-κB activation is important for clearing adenovirus (Gooding 1994; Elkon et al, 1997; Morelli et al, 2000), this interaction may modulate the NF-κB pathway as part of a viral defence strategy (Friedman et al, 2002). ORF E10 of EHV-2 encodes viral CARD-like apoptotic protein (vCLAP) (Srinivasula et al, 1999; Koseki et al, 1999; Costanzo et al, 1999; Thome et al, 1999), the homologue of cellular Bcl-10 (Section 1.3.3.4) (Srinivasula et al, 1999). Like Bcl-10, vCLAP contains two domains, an N-terminal CARD and a novel C-terminal domain (CTD), and can induce both apoptosis and NF-κB activation (Srinivasula et al, 1999; Koseki et al, 1999; Costanzo et al, 1999). vCLAP interacts with the C-terminus of IKKγ via its CTD, inducing persistent activation of IKK (Poyet et al, 2001). The Tax transforming protein of HTLV-1 also physically associates with IKKγ (Fig. 4.1) and activates IKK (Section 1.3.4.4). HTLV-1 is an oncogenic retrovirus etiologically associated with the development of the acute T cell malignancy, adult T-cell leukaemia (Poiesz et al, 1980; Yoshida et al, 1982). As a viral protein capable of activating NF-κB and associated with viral transformation of lymphoid cells, Tax represents the most interesting model for the function of vFLIP.

4.2 Yeast-two-hybrid data.

A yeast-two-hybrid screen was performed by collaborators at Hybrigenics to identify proteins capable of interacting with vFLIP, and 14 IKKγ clones were identified including six independent fusions (Fig. 4.1.C). These data strongly suggested 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 173-269, composed of the third coiled-coil region (CCR3) and the first section of CCR4 (Fig. 4.1.A, B).

4.3 Cloning of IKKγ

IKKγ mutants (Fig. 4.2.A) were constructed with an N-terminal Xpress tag by PCR amplification from an expressed sequence tag and cloning into mammalian expression vector pcDNA 4C (Fig. 4.2.B).

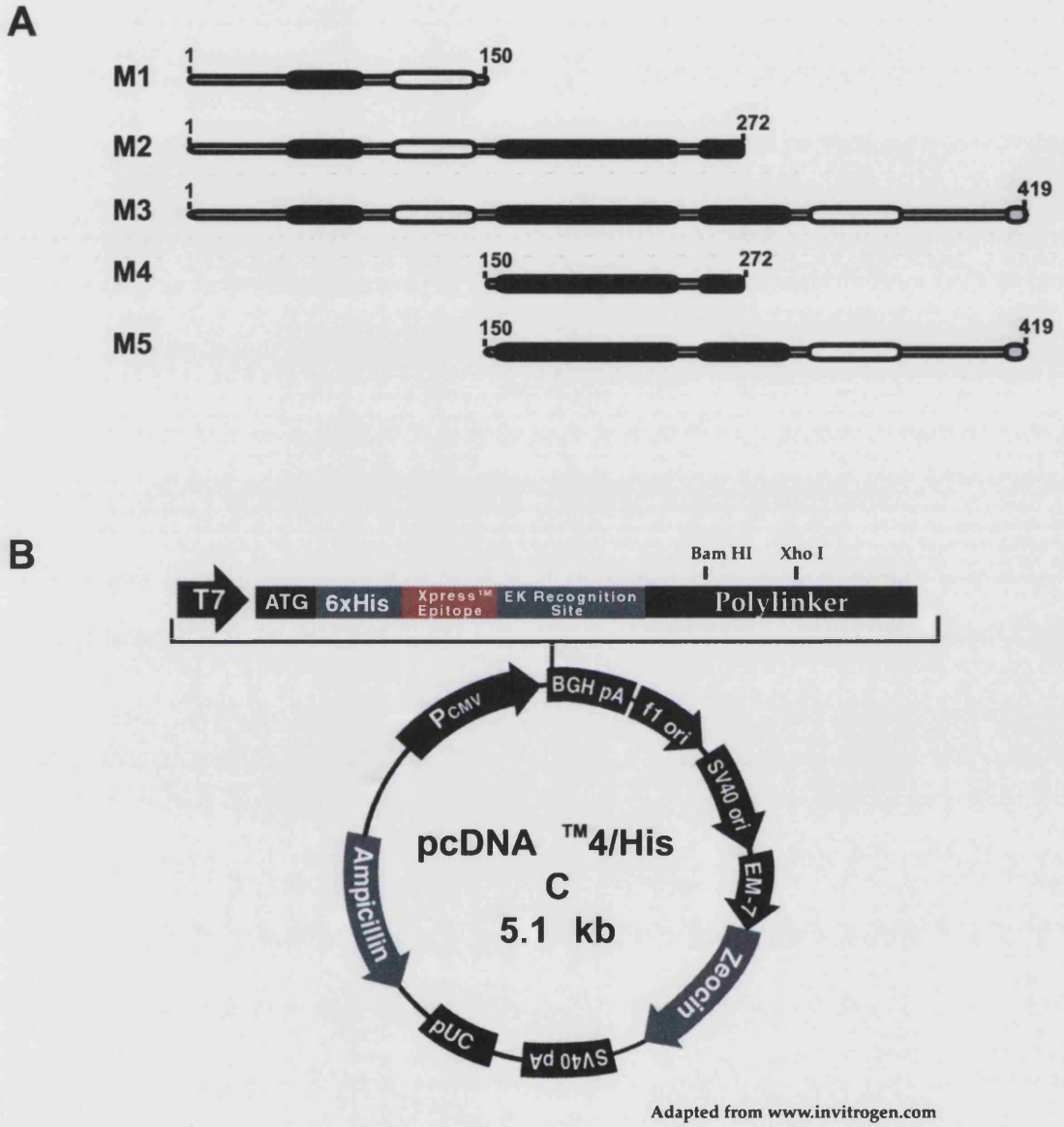


Figure 4.2. Generation of IKKγ truncation mutants.

IKKγ truncation mutants (A) were generated by PCR amplification of a human expressed sequence tag and subsequent cloning of the DNA fragments into the pcDNA4 mammalian expression vector (B) downstream of the Xpress Epitope tag using the Bam HI and Xho I sites indicated.

4.4 vFLIP interaction with IKKγ mutants

Co-immunoprecipitation was used to confirm the domain in IKKγ that mediates its interaction with vFLIP. 293T cells were co-transfected with the IKKγ mutants and with or without vFLIP. Using the anti-vFLIP antibody, Fig. 4.3 shows that all the IKKγ fragments, with the exception of M1 (amino acids 1-150), co-immunoprecipitated with vFLIP. This suggested the minimum interacting domain to be amino acids 150-272 of IKKγ. However, the level of vFLIP in cells co-expressing the M1 mutant was consistently lower (Fig. 4.3 and data not shown). I therefore made the same mutants as GST fusion proteins expressed in bacteria, and examined their ability to interact with vFLIP from equal proportions of transfected 293T cell lysate. Using glutathione coupled sepharose to purify the GST fusions, Fig. 4.4 shows that all the GST-IKKγ fragments, with the exception of M1 bound vFLIP. Thus confirming the minimum vFLIP interacting domain as amino acids 150-272 of IKKγ. It follows that the increased levels of vFLIP observed when vFLIP was co-expressed with mutants containing this domain may be explained by the stabilising effect of this interaction.

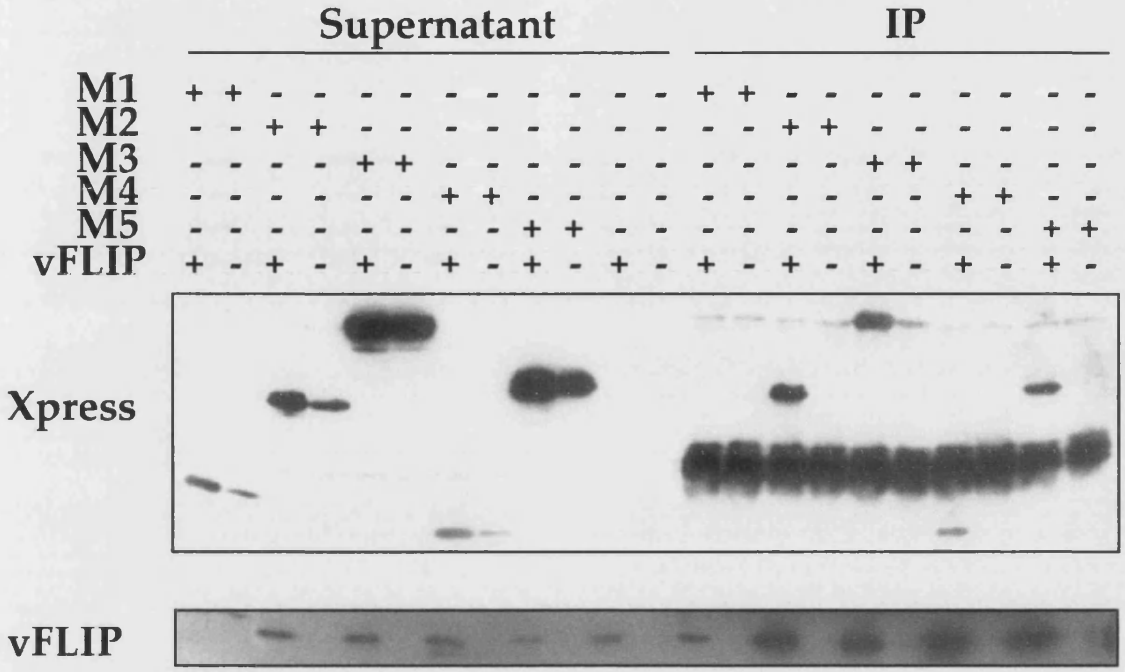


Figure 4.3. Interaction of Xpress tagged IKKγ truncation mutants with vFLIP.

$1x10^6$ 293T cells were co-transfected with vectors (1μg each) encoding IKKγ truncation mutants and either vFLIP or empty vector. Cell lysates (0.2% NP40 buffer) at 48h were immuno-precipitated using the anti-vFLIP antibody and analysed by immunoblot using an anti-Xpress antibody. This gel was representative of three experiments which produced almost identical results.

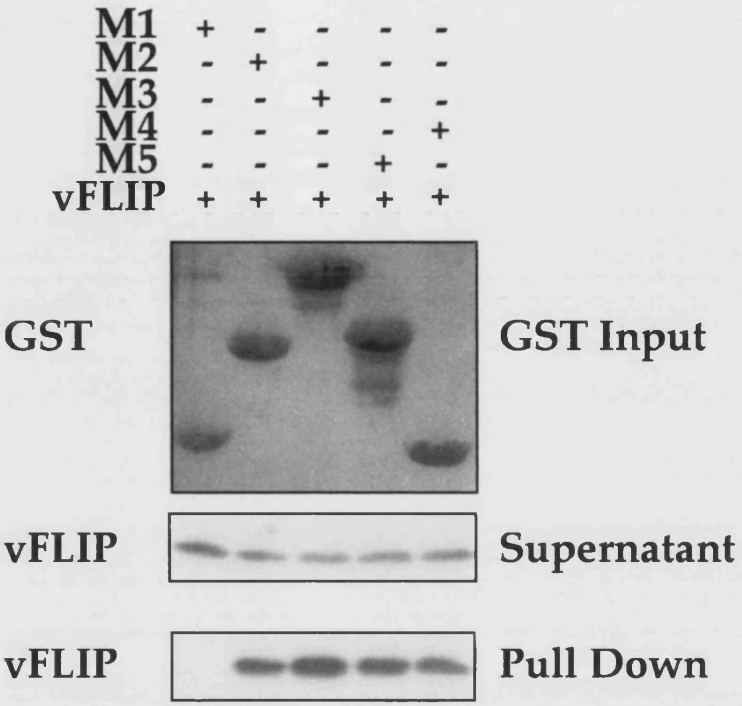


Figure 4.4. Interaction of GST tagged IKKγ truncation mutants with vFLIP.

$1x10^7$ 293T cells were transfected with vFLIP (5μg). GST tagged IKKγ mutants synthesised in *E. coli* were incubated with equal proportions of the 293T cell lysate (0.2% NP40 buffer) for 2h before purification using glutathione sepharose. Co-purification of vFLIP (Pull Down) and 5% of the remaining supernatant (Supernant) were analysed by immunoblot using an anti-vFLIP antibody. The levels of GST-tagged mutants were monitored by Coomassie stain (GST Input). This gel was representative of two experiments which produced almost identical results.

4.5 IKKγ mutants inhibit NF-kB activation

Luciferase reporter assays were used to compare the functional effects of co-expressing vFLIP with either M4 (the vFLIP-interacting domain of IKKγ) or M1. 293T cells were co-transfected with three plasmids: a reporter plasmid containing 3 copies of the κB response element upstream of luciferase, vFLIP, and increasing amounts of the Xpress-tagged mutants or empty vector. Fig. 4.5.A shows that co-expression of both M1 and M4 with vFLIP led to significant and dose-dependent inhibition of NF-κB activity. Since M1 does not interact with vFLIP, it was not expected to inhibit vFLIP. However, M1 contains the IKKα/β binding domain of IKKγ. It may therefore act as a general inhibitor of NF-κB activation, consistent with the ability of the IKKγ-binding domain of IKKα/β to block NF-κB activation (May et al, 2000; May et al, 2002). To test this hypothesis, the luciferase assay was used to examine inhibition of Tax- and TNFα-induced NF-κB activation by M1 and M4. Fig. 4.5.B shows that M1 potently inhibited Tax, while M4 had little influence on NF-κB activation by Tax. The result with TNFα was not as clear; although M1 strongly inhibited NF-κB activation again, M4 did partially reduce NF-κB activation by TNFα (Fig. 4.5.C). These data demonstrate that the M1 IKKγ fragment can inhibit NF-κB activation induced by vFLIP, Tax and TNFα. The M4 fragment appears to act as dominant negative inhibitor of vFLIP-induced NF-κB activation with some degree of specificity. The ability of the N-terminal domain of IKKγ to inhibit NF-κB activation in reporter based assays was recently confirmed by another group (Tegethoff et al, 2003).

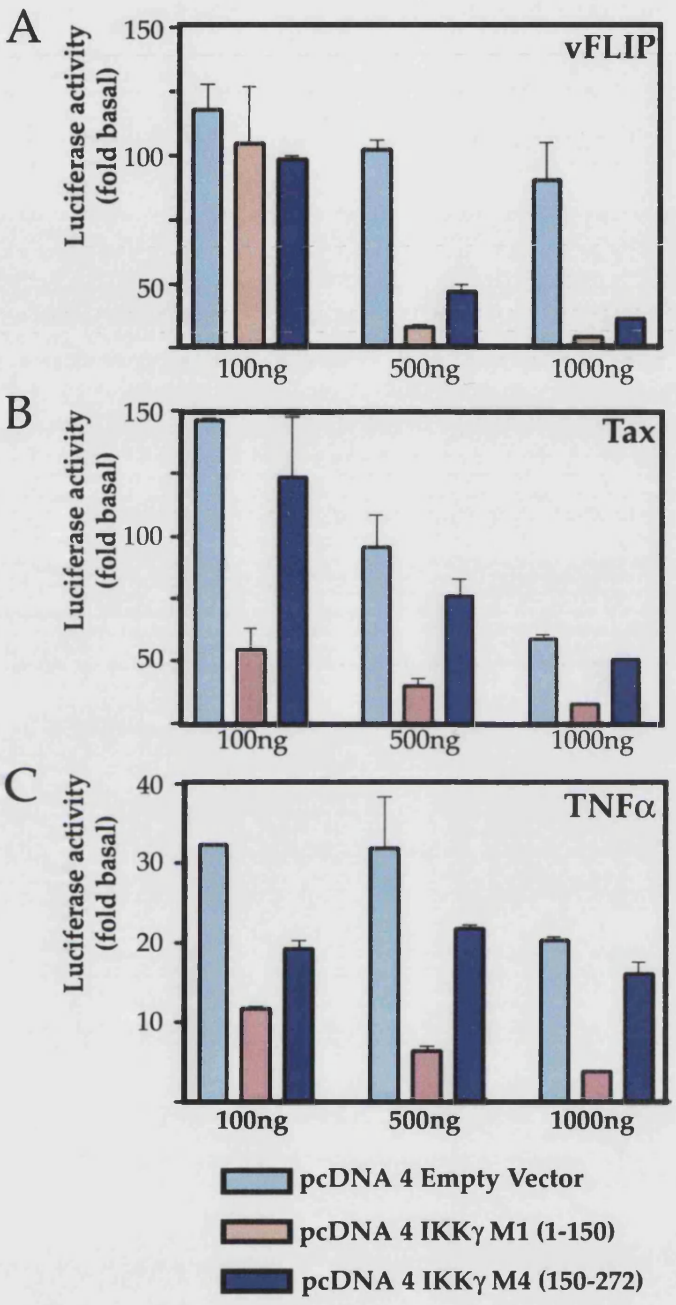


Figure 4.5. IKKγ mutants inhibit vFLIP-induced NF-κB activation.

$1x10^5$ 293T cells were co-transfected with a luciferase reporter plasmid (80ng) containing four NF-κB binding sites upstream of the luciferase gene and varying amounts of IKKγ mutants M1 (1-150), M4 (150-272) or empty vector. Cells were also transfected with 0.2μg of either vFLIP (A) or Tax (B) or stimulated with 10ng/ml TNFα for 2h (C) to activate NF-kB. Luciferase activity was measured by luminometer at 48h post-transfection. The data are plotted as fold basal activity in mock-transfected controls. Error bars are calculated on duplicate samples.

4.6 IKKγ mutants compete with endogenous IKKγ

To test whether the IKKγ mutants compete with endogenous IKKγ for binding to vFLIP, 293T cells were co-transfected with vFLIP and either empty vector, M1, M2, M4 or M5. The anti-vFLIP antibody was used to immunoprecipitate vFLIP, and immune complexes were analysed for the co-purification of endogenous IKKγ. Fig. 4.6 shows that co-purification of endogenous IKKγ was blocked when vFLIP was co-expressed with all mutants containing amino acids 150-272. Since M4 lacks the IKKα/β binding domain, this fragment may sequester vFLIP in a complex that cannot be activated. This may explain the mechanism by which M4 blocks vFLIP-induced NF-κB activation (Fig. 4.5.A). Although the association between endogenous IKKγ and vFLIP was not disturbed by M1, the level of vFLIP expression was consistently reduced whenever co-expressed with M1 in comparison to empty vector. This observation could not be explained.

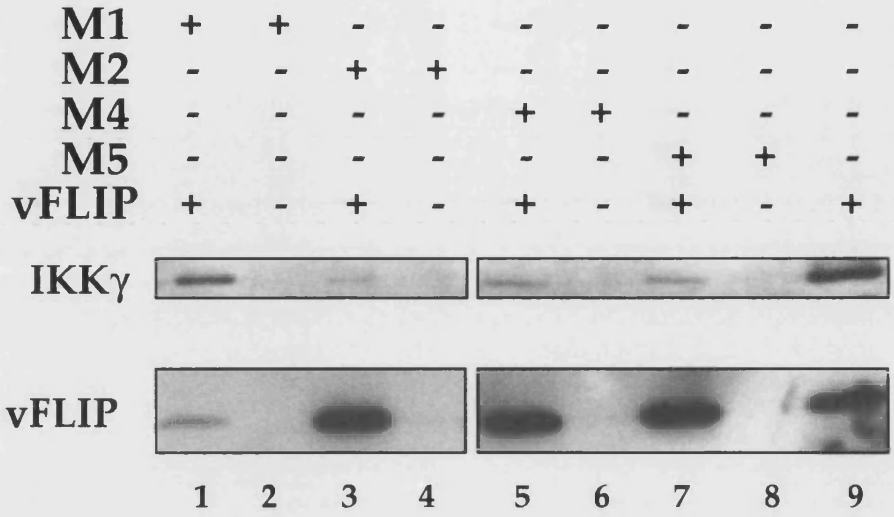


Figure 4.6. IKKγ mutants containing amino acids 150-272 compete with endogenous IKKγ for binding to vFLIP.

$1x10^6$ 293T cells were co-transfected with expression vectors (1μg each) encoding IKKγ truncation mutants (M1, M2, M4 and M5) or empty vector and either vFLIP or empty vector. Cell lysates (0.2% NP40 buffer) at 48h were immunoprecipitated using an anti-vFLIP antibody and analysed by immunoblot for co-purification of endogenous IKKγ.

4.7 IKKγ-deficient Jurkat cells

4.7.1 8321 cells express a truncated IKKγ protein

Jurkat 3T8 T cells and a 3T8 derivative with a reported deficiency in IKKγ (Section 2.4.1), termed 8321, were used to examine the role of IKKγ in vFLIP-induced activation of IKK and NF-κB. In summary, to derive the 8321 cells, the 3T8 cells were sequentially transfected by He and colleagues, with two reporter genes to express human CD14 and rat Thy1 under control of the NF-κB enhancer element, and mutagenesis was performed using the alkylating agent, ICR191 (Ting et al, 1996). Clonal cell lines with defects in the NF-κB pathway were obtained from the pool of negatively enriched cells that failed to express CD14 or Thy1 (He et al, 2002). The 8321 clone failed to activate NF-κB in response to TNFα, phorbal myristate acetate (PMA) and phytohaemagglutinin (PHA). A monoclonal antibody raised against His-tagged full-length IKKγ (Imgenex IMG-324) was used to demonstrate loss of IKKγ expression in 8321 cells (He et al, 2002). However, using a polyclonal rabbit antibody raised against the full-length IKKγ protein (Santa Cruz SC-8330), a truncated species of IKKγ was observed to associate with vFLIP when the 8321 mutant was infected with lentivirus encoding vFLIP (Fig. 4.7). The association between vFLIP and full length IKKγ in BC3 cells and lentivirus-infected 3T8 cells is also shown. The discrepancy between these data is probably explained by the use of different antibodies to detect IKKγ.

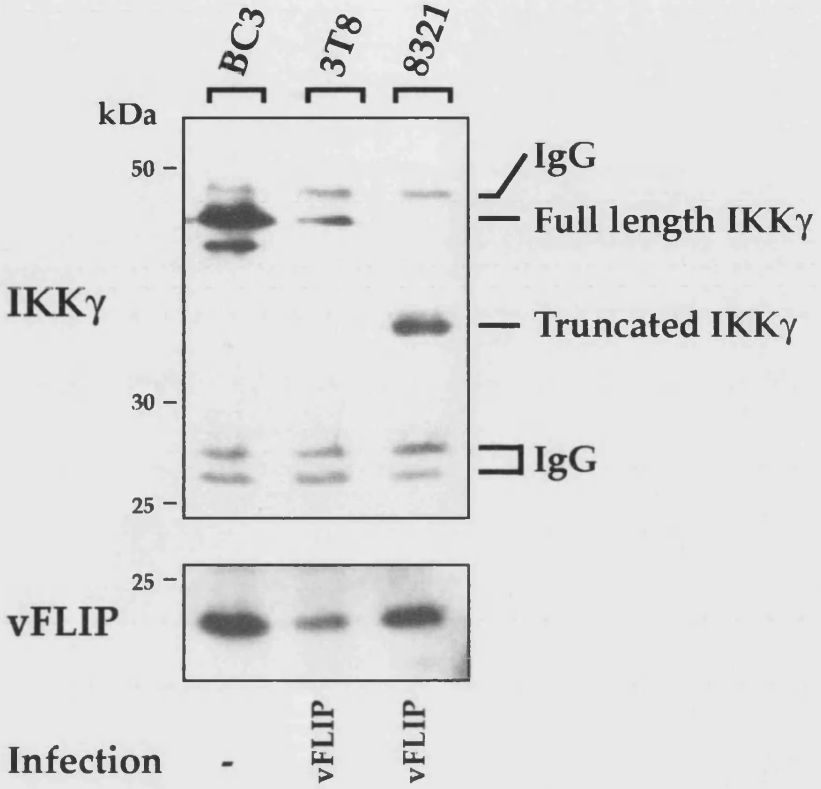


Figure 4.7. vFLIP interacts with a truncated species of IKKγ in Jurkat cells with deficient NF-κB signalling.

BC3 cells, parental Jurkat Cells (3T8) and a Jurkat derivative (8321) previously reported to have a deficiency in IKKγ were used in this experiment. Both Jurkat 3T8 and Jurkat 8321 cells were infected with a lentivirus encoding vFLIP plus GFP. $1x10^7$ cells from each cell line were lysed (0.2% NP40 buffer), lysates were then immunoprecipitated using the anti-vFLIP antibody. 50% of the immunoprecipitated protein was analysed by immunoblot using anti-IKKγ and anti-vFLIP antibodies. The remaining 50% was assayed for IκBα kinase activity (see Figure 4.8). Cross reacting IgG bands are labelled (IgG).

4.7.2 Functional activity of vFLIP in IKKγ-mutant Jurkat cells

The observation that the 8321 cells express a truncated IKKγ fragment was unexpected. Kinase assays and electrophorectic mobility shift assays (EMSAs) were therefore used to examine the activation of NF-κB by vFLIP in these cells. Kinase assays were performed using the anti-vFLIP antibody to isolate the vFLIP-complex from lentivirus-infected 3T8 and 8321 Jurkats and from BC3 cells. Kinase activity associated with vFLIP was found in each cell line (Fig. 4.8), although the level of kinase activity was substantially greater in BC3 cells. This may be attributed to the relative levels of vFLIP expression (Fig. 4.7). The vFLIP immune complex in each cell line was not able to phosphorylate a mutant GST-IκBα substrate, confirming the substrate specificity of this assay.

EMSAs were performed on the nuclear extracts of BC3, 3T8 and 8321 cells to assess downstream activation of the NF-κB pathway and confirm proper signal transduction. Fig. 4.9 shows that in both 3T8 and 8321 Jurkats, a major NF-κB binding complex was induced by vFLIP expression. An identical shifted band is found in the BC3 track (Fig.4.9). The specificity of κB binding was analysed using a mutant κB oligonucleotide probe, and by competition with 100-fold unlabelled κB oligonucleotide. These data indicate that the IKKγ truncation found in 8321 Jurkat cells does not affect the ability of vFLIP to activate the NF-κB pathway.

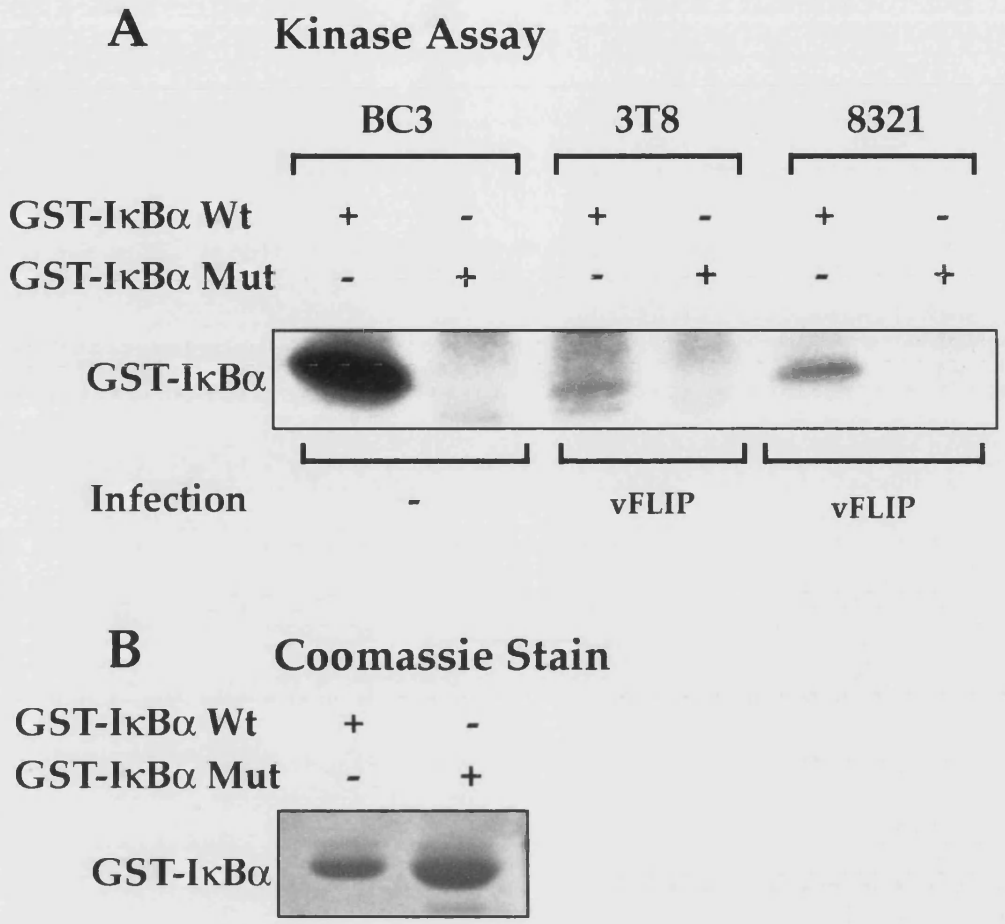
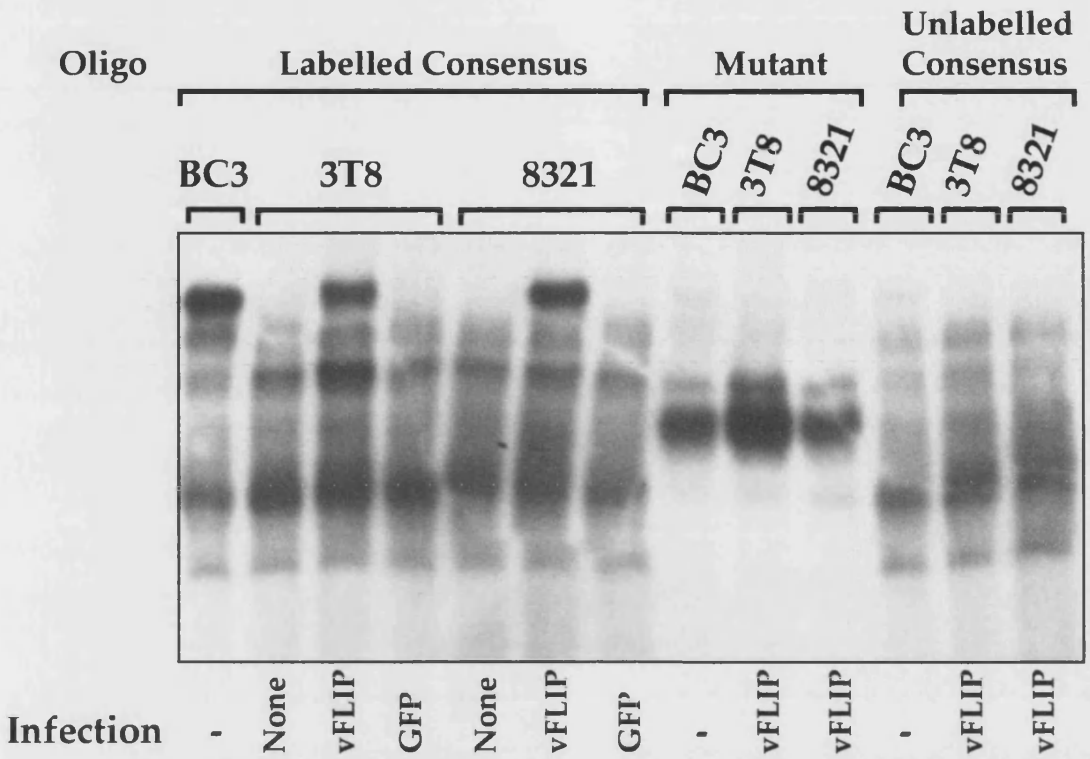


Figure 4.8. vFLIP activates IKK in Jurkat 3T8 and 8321 cells.

(A) Cell lysates (0.2% NP40 buffer) from BC3, and Jurkat 3T8 and 8321 cells infected with a lentivirus encoding vFLIP plus GFP ($1x10^7$cells) were immunoprecipitated using the anti-vFLIP antibody (see Figure 4.7). The immune complexes were incubated with either wildtype or mutant (S32A/S36A) GST-IκBα substrates, and labelled proteins were detected by autoradiography. The levels of GST-tagged substrates were monitored by Coomassie stain (B).

A



B

Consensus

```
5'- AGT TGA GGG GAC TTT CCC AGG C -3'
3'- TCA ACT CCC CTG AAA GGG TCC G -5'
```

Mutant

```
5'- AGT TGA GGC GAC TTT CCC AGG C -3'
3'- TCA ACT CCG CTG AAA GGG TCC G -5'
```

Figure 4.9. vFLIP activates nuclear proteins that bind kB consensus response elements in Jurkat 3T8 and 8321 cells.

(A) EMSAs were performed using total nuclear extracts (7.5µg per track) prepared from BC3 and from Jurkat (3T8 and 8321) cells either uninfected or infected with lentivirus encoding vFLIP plus GFP or GFP alone. NF-κB binding activity was determined using a γ-P^{32}-labelled oligonucleotide probe containing the κB response element (labelled consensus) and specificity was monitored using a labelled control probe (mutant) and by competition with 100-fold unlabelled consensus oligonucleotide (unlabelled consensus). (B) The sequences of the consensus and mutant oligonucleotides are shown. The single base pair substitution of the mutant probe is marked.

4.8 Discussion.

This chapter focuses on the role of IKKγ in vFLIP activation of the NF-κB pathway. The central region of IKKγ, between amino acids 150 and 272, is shown to be essential for the vFLIP interaction. This is in contrast to the leucine zipper regions required for the Tax-IKKγ interaction (Fig. 4.1.A). Deletions of LZ1 abolish the binding of Tax to IKKγ, whereas mutants lacking LZ2 show reduced binding to Tax (Xiao et al, 2000). The vFLIP binding region does not overlap with either region. From these data it can be inferred that the structurally unrelated viral proteins Tax and vFLIP have evolved distinct mechanisms to bind IKKγ and thereby activate IKKγ.

vFLIP is also shown to activate NF-κB in a mutant Jurkat cell line, 8321, known to be refractory to stimulation by TNFα, PMA and PHA (He et al, 2002). These cells are shown to express a truncated IKKγ protein that associates with vFLIP and supports normal levels of IKK activity and NF-κB DNA binding activity. The same cells had previously been used to show that A20 could protect from TNFα-mediated apoptosis (Section 4.1.4), apparently in the absence of IKKγ (He et al, 2002). These data were interpreted to suggest that the A20-IKKγ interaction is not required for A20-mediated protection against apoptosis. The data presented here cast doubt on this explanation. Since vFLIP-induced NF-κB activity appears to be normal in 8321 cells, the implication is that A20 might also activate IKK and thus protect these cells from TNFα-mediated apoptosis. He et al, did not, for example, assay for A20-associated IKK activity or for NF-κB activation in A20-transfected cells. The possibility that A20 may also activate IKK in 8321 cells cannot be ruled out.

Although currently a matter for speculation, the IKKγ mutant expressed by 8321 cells is most likely to contain a C-terminus truncation because the N-terminus and intermediate domains are probably intact. This inference is made because the N-terminus contains the IKKα/β-binding domain and is required for IKK activation, and the intermediate domain is required for association with vFLIP. Interestingly, this implies that vFLIP may not require the IKKγ C-terminus for activation of IKK. This

is in direct contrast to a number of other known stimuli. Cloning of this truncated IKKγ protein will reveal the location of the genetic mutation and may shed light on the mechanism of vFLIP-induced IKK activation. With further experimentation, it may be possible to define the minimum domain of IKKγ that is required for vFLIP-induced IKK activity.

CHAPTER 5

vFLIP induces p100 processing

5.1 Introduction

The alternative pathway of NF-κB activation induces the processing of p100 to generate p52 (Section 1.3.4.1). This pathway involves the phosphorylation of p100 via NIK and IKKα, leading to its ubiquitylation through the SCF ubiquitin ligase complex and subsequent processing to p52 (Sections 1.3.4.1; 1.3.4.2). This process is tightly regulated (Xiao et al, 2001b) for good reason; chromosomal translocations at the 10q24 locus that truncate the C-terminus of p100 leading to aberrant processing are associated with lymphomas (Fracchiolla et al, 1993; Thakur et al, 1994; Chang et al, 1995), and genetically manipulated mice expressing p52, but not p100, develop gastric and lymphoid hyperplasia (Ishikawa et al, 1997). Physiological stimuli that regulate the processing of p100 to p52 include BAFF ligand, CD40 activation, lymphotoxin β, lipopolysaccharide and TWEAK (Section 1.4.3.3). Without exception, all have important roles in the development and regulation of the immune system, and in particular B cell function. It is therefore of great interest that deregulated p100 processing has been found in leukaemic T cells transformed by HTLV-1, in which Tax induces the processing, and that the EBV transforming protein, LMP1, is also able to induce p100 processing (Section 1.3.4.4). Constitutive NF-κB activation within infected lymphoid cells is central to the transforming activity of both viral oncoproteins (Section 1.3.4.4). As a constitutive activator of NF-κB expressed by a lymphotropic and oncogenic virus, I speculated that vFLIP might also activate this alternative pathway.

5.2 DNA input affects the processing of p100 but not p105

Although the processing of p105 is largely constitutive (Lin et al, 1998; Lin et al, 2000), constitutive processing of p100 is limited. However, upon expression of exogenous p100 in 293T cells, significant p52 generation was observed in the absence of any stimuli (Fig. 5.1 and data not shown). To assess the contribution of overexpression to this observation, the quantity of transfected p100 and p105 expression vectors was titrated. As expected, higher levels of input DNA led to increased expression of both p100 and p105 (Fig. 5.1). Fig. 5.1.A shows that p52 was undetectable when 0.1μg of p100 was transfected per well. With 0.5μg input DNA per well, p52 was observed, although at a lower level than p100. A further increase in the amount of transfected DNA did not increase the level of p52 (Fig. 5.1.A). By contrast, the ratio of p50 to p105 was close to 1:1 at all levels of input DNA (Fig. 5.1.B). These data demonstrate that processing of exogenous p100 can be associated with overexpression. For subsequent experiments, the level of p100 input was therefore maintained at 0.1μg per well, unless otherwise stated.

5.3 vFLIP induces p52 production in transfected 293T cells

To examine whether vFLIP regulates the processing of p100 to p52, 293T cells were co-transfected with p100 and either vFLIP, empty vector or Tax. For comparison, a similar experiment was performed using p105. Fig. 5.2.A shows that, in the absence of vFLIP or Tax, p100 was clearly expressed but no p52 was detected. As expected, Tax expression induced a significant increase in p52 levels (Xiao et al, 2001a). Transfection of vFLIP led to a similar increase in p52 levels. Co-expression of vFLIP with p100 therefore stimulates p52 accumulation. However, because p100 levels were not affected by either vFLIP or Tax expression, it is not possible to say whether this is due to increased p100 processing. By contrast, co-expression of vFLIP with p105 did not increase the accumulation of p50 (Fig. 5.2.B). Interestingly, since these experiments were performed, it has been reported that $cFLIP_L$ both associates with and prevents the processing of p105 (Li et al, 2003). Whether these observations are related to the effect of vFLIP on p100/p52 is not yet clear.

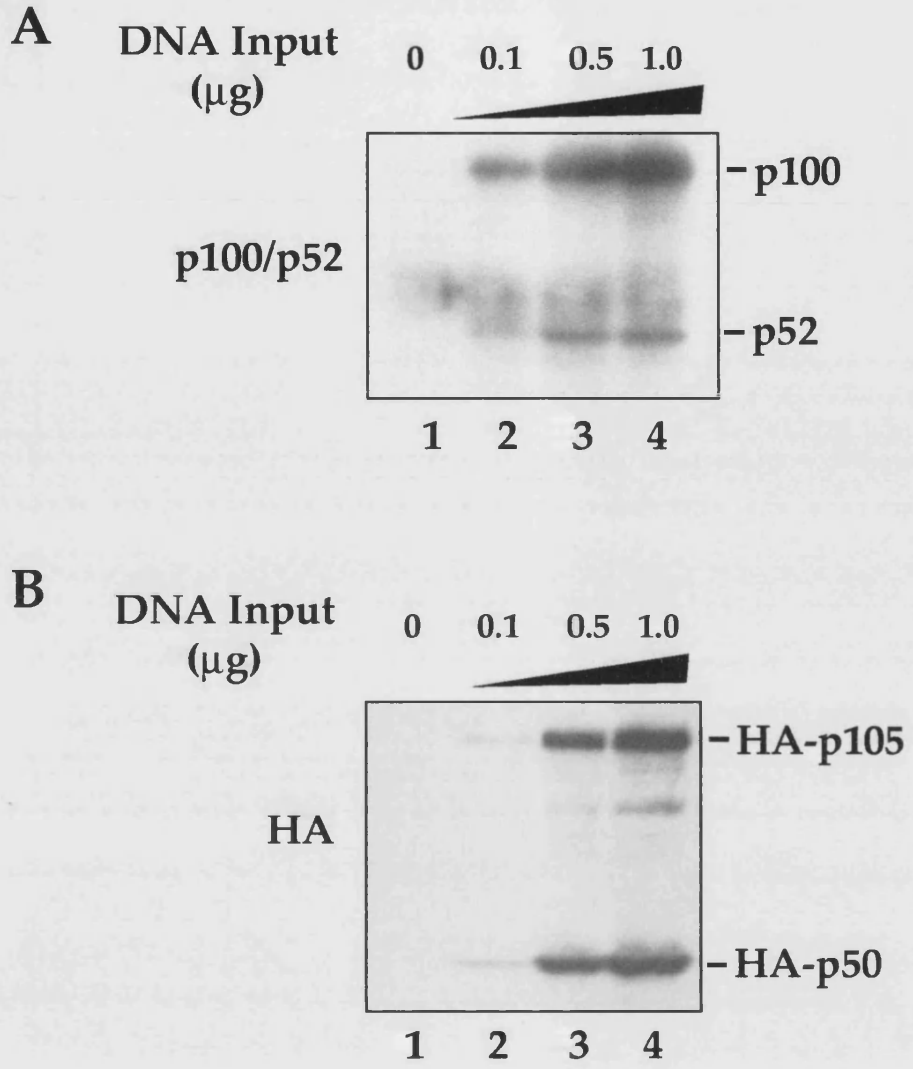


Figure 5.1. The affect of varying the DNA plasmid input on constitutive processing of p100 and p105.

$1x10^6$ 293T cells were transfected with 0, 0.1, 0.5 or 1.0 μg of pcDNA3.1 encoding either p100 (A) or HA-p105 (B). Cell lysates (RIPA buffer) at 48h were assayed for protein concentration by the Bradford method and 20μg of lysate were loaded in each track. The processing of p100 to p52, and of p105 to p50 was analysed by immunoblotting using a rabbit polyclonal anti-p100 antibody raised against residues 1-15 of human p100 (See Table 2.3), and an anti-HA antibody as indicated.

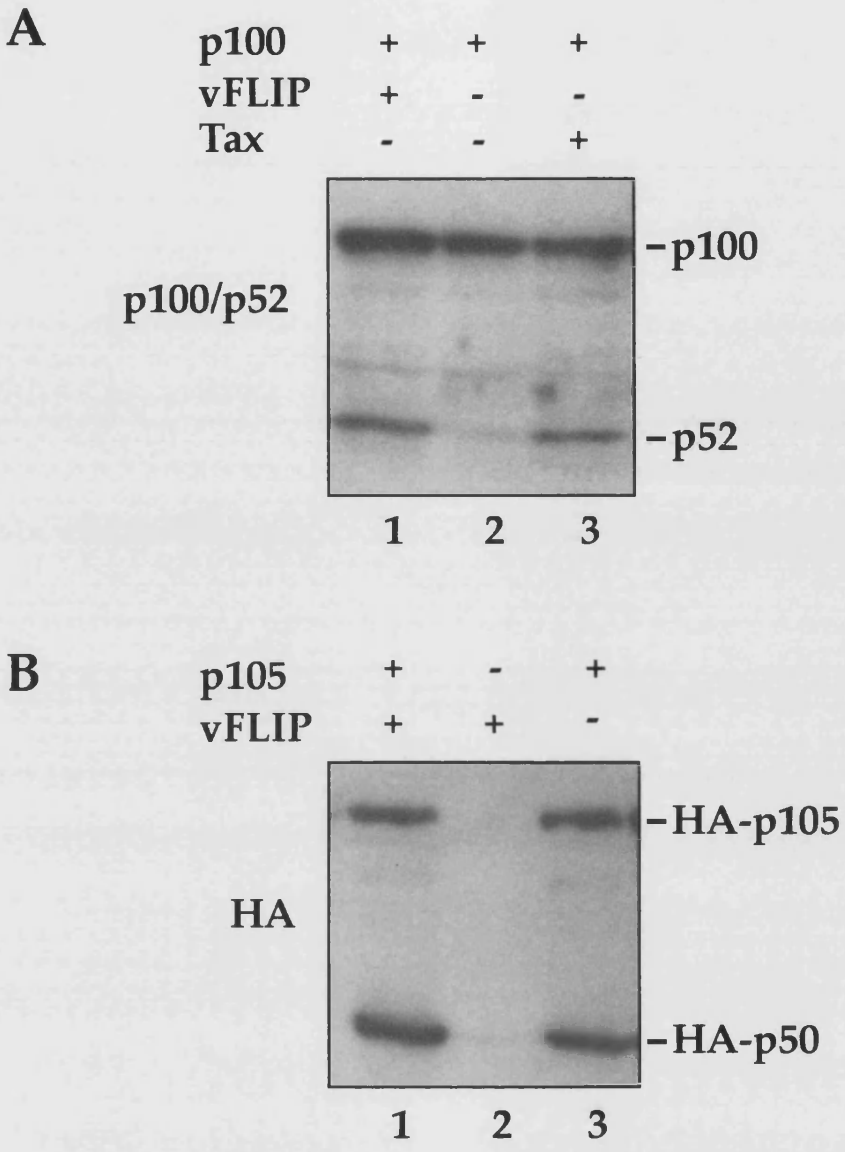


Figure 5.2. Co-expression of vFLIP increases the generation of p52, but not of p50.

$1x10^6$ 293T cells were co-transfected with expression vectors encoding p100 (0.1µg) and either vFLIP, Tax or empty vector (1µg each) (A) and with HA-p105 (0.1µg) and either vFLIP or empty vector (1µg) (B). 20ug of lysate (RIPA buffer) at 48h was analysed by immunoblotting using a commercial mouse monoclonal anti-p100 antibody generated using GST fused to residues 1-444 of human p100 (See Table 2.3), and an anti-HA antibody as indicated.

5.4 vFLIP-induced p52 accumulation requires p100 serines 866/870

Processing of p100 to p52 by NIK (Xiao et al, 2001b), CD40 (Coope et al, 2002) and Tax (Xiao et al, 2001a) requires two serines (S866 and S870) in the C-terminus of p100 (Fig. 5.3.A). To determine whether these residues are required for vFLIP-induced p52 accumulation, 293T cells were co-transfected with either wild-type p100 or a mutant p100-S866A/S870A containing serine to alanine mutations at these residues and vFLIP, Tax or empty vector. vFLIP induced a reproducible increase in levels of exogenous p52 in wild-type-p100-transfected cells but not in cells transfected with p100-S866A/S870A (Fig. 5.3.B). This implies that serines 866 and 870 of p100 are required for the vFLIP-induced production of p52. In the context of previous reports that the phosphorylation of p100 by IKKα at these serine residues is associated with p100 ubiquitylation and processing, these data are consistent with, but do not prove, vFLIP-induced processing of p100.

5.5 vFLIP physically associates with p100

The mechanism of Tax-induced p100 processing has been partially explained by the ability of Tax to bind p100 (Beraud et al, 1994) and recruit it to the IKK complex (Xiao et al, 2001a). Since vFLIP, like Tax, binds directly to IKKγ (Chapter 4), it was an intriguing possibility that vFLIP might also contact p100. Co-immunoprecipitation was used to examine whether vFLIP interacts with p100. 293T cells were transfected with 1μg of p100 (wild-type p100 or p100-S866A/S870A) and either vFLIP, Tax or empty vector, and the polyclonal anti-p100 antibody was used to immunoprecipitate p100. Fig. 5.4 demonstrates that vFLIP and Tax both physically associate with wild-type p100 and the mutant p100-S866A/S870A. Although less than 5% of vFLIP was routinely observed to interact with p100 in this overexpression system, vFLIP was not immunoprecipitated by the antibody alone. These data indicate that vFLIP and p100 can physically associate, and that the association does not depend upon serines 866 and 870.

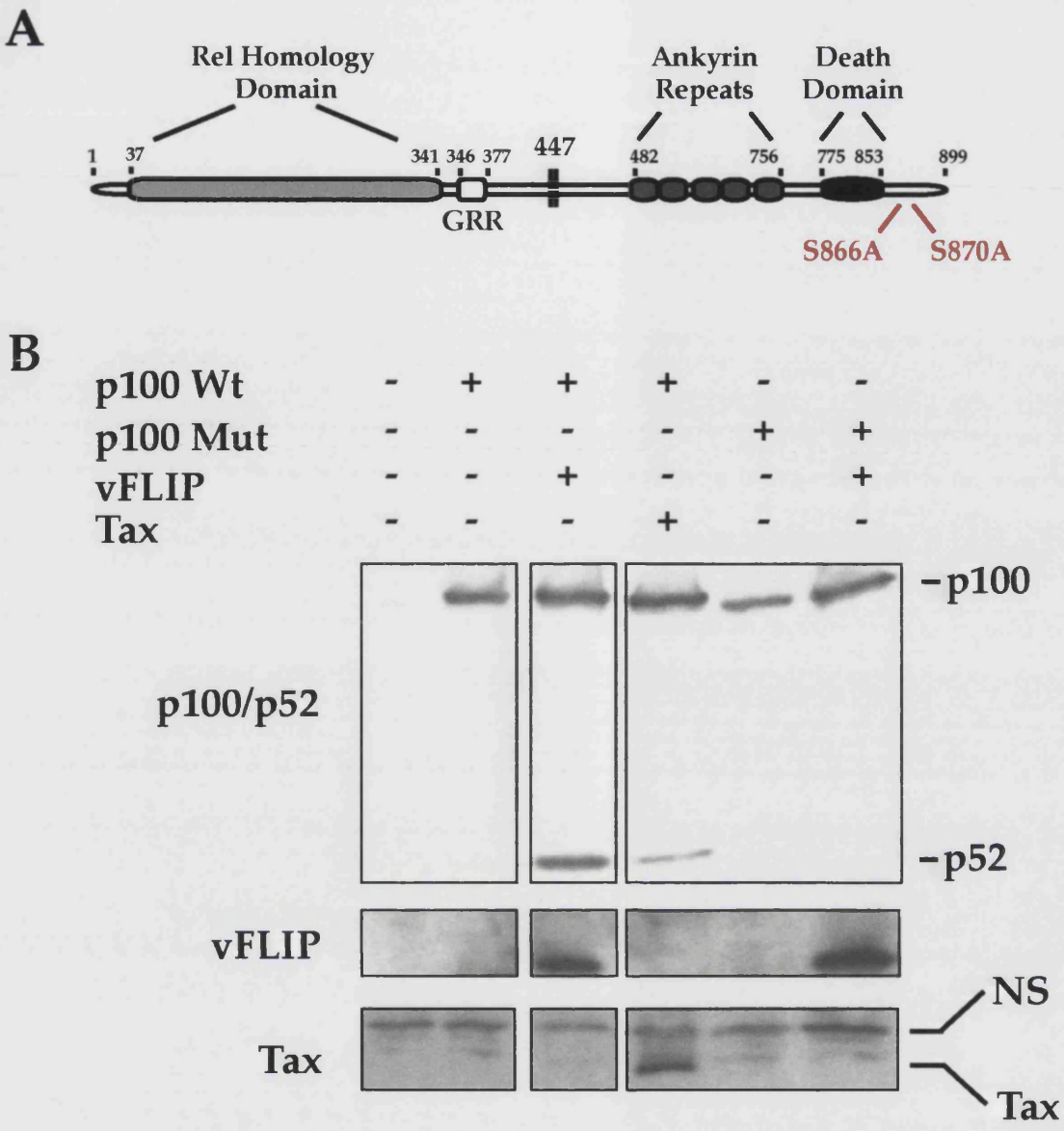


Figure 5.3. vFLIP-induced p52 accumulation requires p100 serines 866 and 870.

A) Schematic picture of p100. The two serine residues (S866A and S870A) in the C-terminus of p100 required for NIK-induced processing to p52 are indicated.
B) $1x10^6$ 293T cells were co-transfected with vectors encoding vFLIP or Tax (1µg each) and p100 wildtype (Wt) or p100 S866A/S870A (Mut) (0.1µg each). 20µg of cell lysate (RIPA buffer) at 48h were immunoblotted using the monoclonal anti-p100/p52, anti-vFLIP and anti-Tax antibodies. NS indicates a non-specific band.

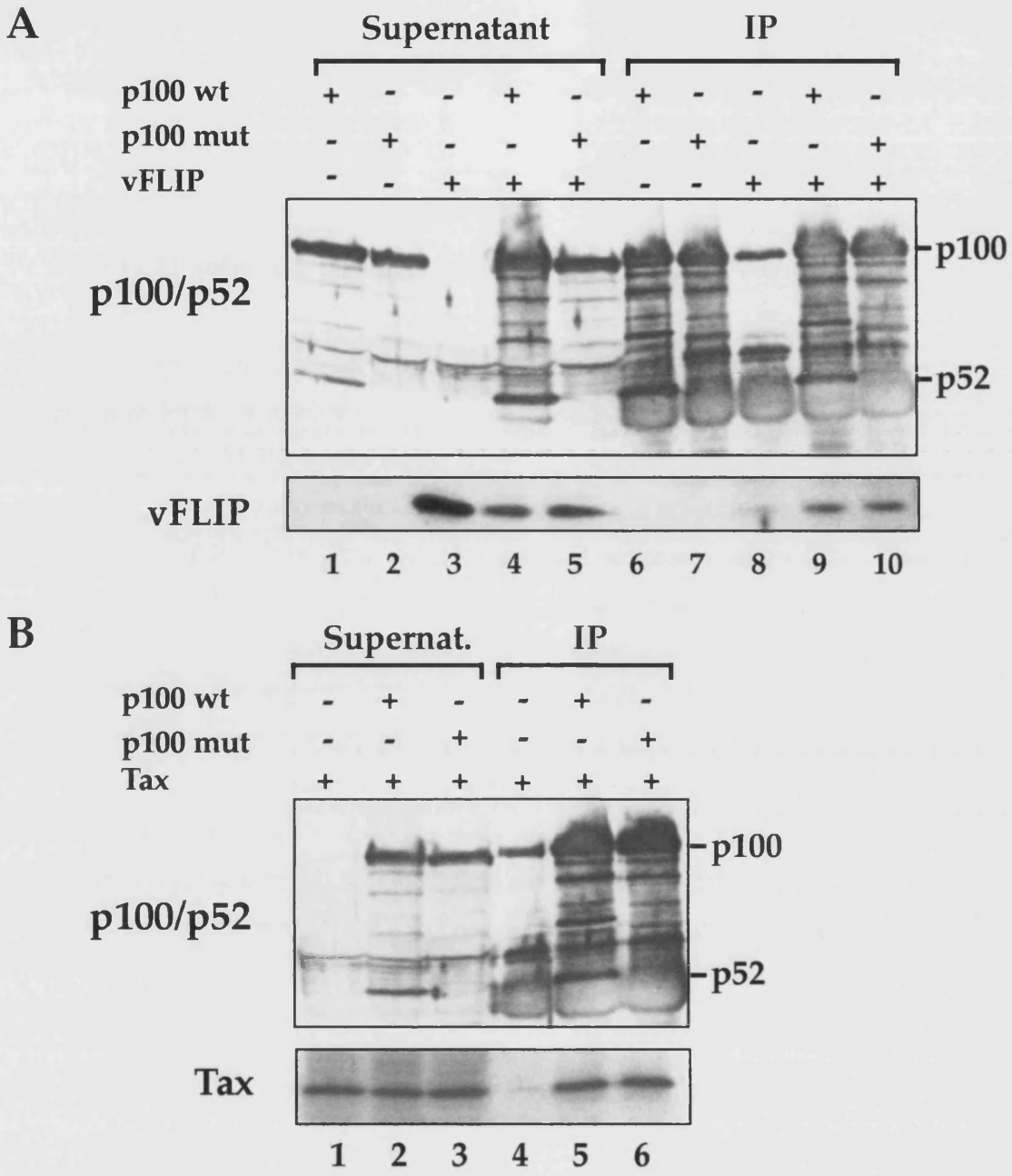


Figure 5.4. Interaction of vFLIP with p100

$1x10^6$ 293T cells were co-transfected with 1µg of indicated p100 expression vectors (Wt or Mut S866A/S870A) and either vFLIP (A) or Tax (B) (1µg each). Cell lysates (0.2% NP40 buffer) at 48h were immunoprecipitated using the rabbit polyclonal anti-p100/p52 antibody. Immune complexes and 5% of the remaining supernatant were analysed by immunoblot using the monoclonal anti-p100/p52, anti-vFLIP and anti-Tax antibodies. Low levels of endogenous p100 were isolated by IP (A - lane 8; B - lane 4).

5.6 vFLIP interacts with p100 in BC3 cells

Expression of p100 is upregulated during B cell development (Senftleben et al, 2001) and high levels of p100 are found in many mature B cell lines (Liou et al, 1994). Generation of p52 also occurs during B cell maturation (Claudio et al, 2002; Coope et al, 2002), and p52 accumulation is found in a variety of B cell lymphomas (Hacker et al, 2002). Fig 5.5 shows that both p100 and p52 are present in the KSHV-infected BC3 cell line. Fig. 5.5 also demonstrates the interaction between endogenous vFLIP and p100. A small proportion of vFLIP was observed to associate with p100. This is in contrast to the majority of vFLIP that associates with IKKγ (Fig. 3.3A). The association between Tax and p100 is thought to recruit p100 to the IKK complex (Xiao et al, 2001a), however it was not possible to determine whether the p100-associated vFLIP was also bound to IKKγ (data not shown). These data demonstrate the expression of both vFLIP and p100 in the context of KSHV infection, and show the interaction between these two proteins under physiological conditions.

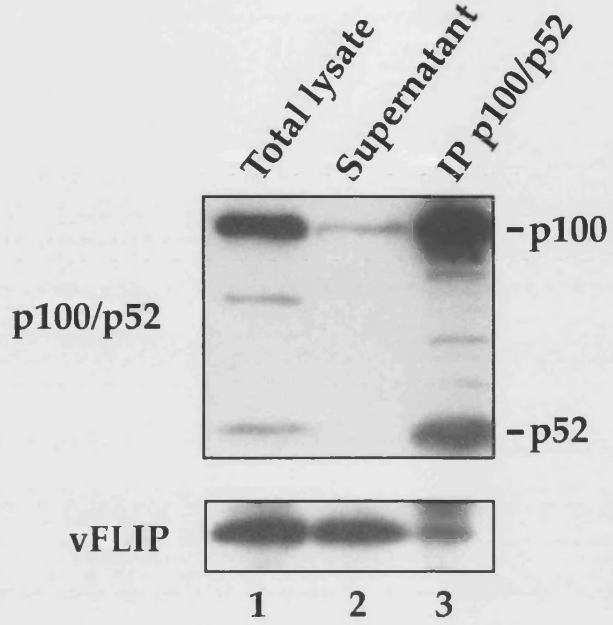


Figure 5.5. Physical association of endogenous p100 and vFLIP.

Cytoplasmic extract (0.2% NP40 buffer) from $5x10^6$ BC3 cells was immunoprecipitated using the polyclonal anti-p100/p52 antibody. The immunoprecipitate (IP p100/p52) and 5% of both the original extract (Total Lysate) and the supernatant from the immunoprecipitation (Supernatant) were then analysed by immunoblot using the anti-vFLIP and the monoclonal p100/p52 antibodies. See Figs. 3.3.A and 5.4.B for comparison.

5.7 vFLIP induces p100 expression and p52 generation in Jurkat cells

To avoid the constitutive p100/p52 expression found in many B cell lines, Jurkat 3T8 T cells (Section 2.4.1) were used to investigate vFLIP induction of p100 processing in a lymphoid cell line. Xiao et al, demonstrated that both Tax and mitogen stimulation increased p100 expression in Jurkat cells, however only Tax led to increased p52 generation (Xiao et al, 2001a). Jurkat 3T8 cells were therefore infected with lentivirus encoding either vFLIP and GFP or GFP alone, followed by analysis of endogenous p52 generation. Expression of vFLIP led to a significant increase in p100 expression and to the accumulation of p52 (Fig. 5.6). The aberrant expression of p100 and p52 was also detected in the Jurkat 8321 cell line that expresses a truncated IKKγ (Figs. 4.7; 5.6). The increase in p100 expression is consistent with previous studies demonstrating the NF-κB2 gene as an NF-κB target (Liptay et al, 1994; Sun et al, 1994). In these studies, activation of NF-κB was associated with p100, but not p52 expression. Low levels of p100 and undetectable p52 expression were observed in uninfected and GFP-infected cells. The generation of p100 indicates activation of the classical pathway by vFLIP, while the accumulation of p52 suggests p100 processing (Section 1.4.3.3). These data are therefore consistent with the activation of both classical and alternative pathways of NF-κB by vFLIP.

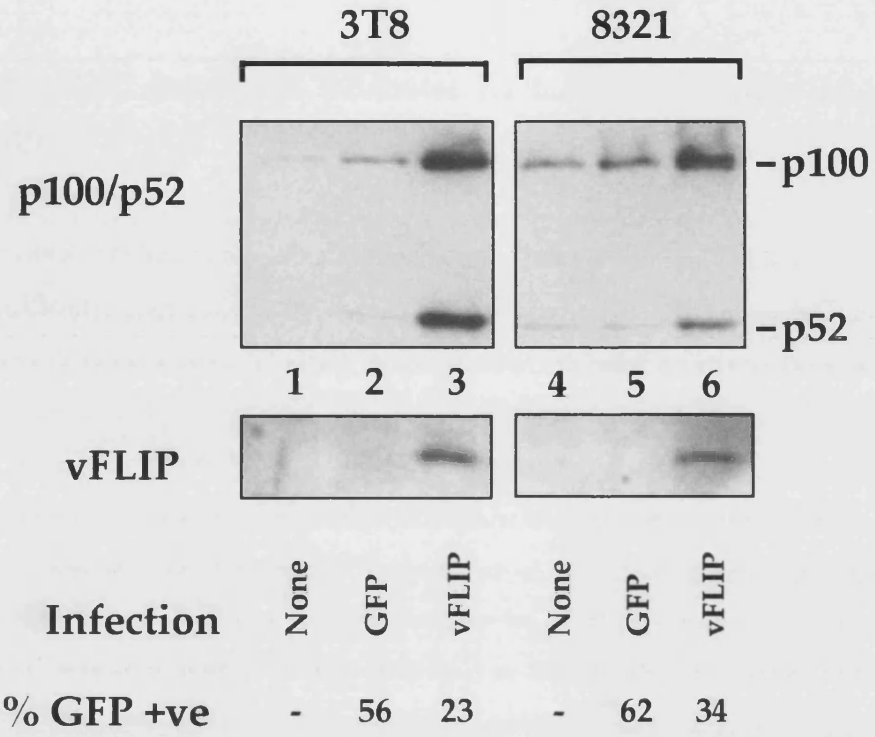


Figure 5.6. vFLIP induces p100 expression and p52 generation in Jurkat 3T8 and 8321 cells.

$5x10^6$ parental Jurkat (3T8) or a Jurkat derivative (8321) encoding a truncated IKKγ were either not infected or transduced with a lentivirus encoding either GFP alone or vFLIP plus GFP. 20μg of cell lysate (RIPA buffer) was analysed by immunoblot using the monoclonal anti-p100/p52 and anti-vFLIP antibodies. Transduction efficiency was measured by FACScan analysis of cells expressing GFP.

5.8 vFLIP expression promotes nuclear translocation of p52 and RelB

Previous studies have demonstrated that NF-κB dimers containing RelB are specifically sequestered in the cytoplasm by p100 (Solan et al, 2002). The transcriptional activity of RelB is specifically inhibited by p100 (Dobrzanski et al, 1995), and RelB is retained in the cytosol of breast cancer cell lines by p100 (Dejardin et al, 1995). Solan et al, further demonstrated that RelB was associated in the cytoplasm with p100 and not other IκB molecules, and that p100 processing resulted in nuclear translocation of RelB (Solan et al, 2002). It follows that the nuclear translocation of RelB is a surrogate marker for p100 processing. Immunoblotting of nuclear and cytoplasmic extracts from BC3 or Jurkat 3T8 cells confirmed that vFLIP expression is associated with an increase in nuclear RelB (Fig. 5.7). Levels of nuclear p52 were also found to increase with the expression of vFLIP in 3T8 cells, although levels of nuclear RelB and p52 were significantly higher in BC3 cells (Fig. 5.7). This may be attributable to the lower levels of vFLIP expression in 3T8 cells (Fig. 5.6). Immunoblotting for tubulin (cytoplasmic marker) and Sp1 (nuclear marker) confirmed cell fractionation and protein loading. These data imply that vFLIP induces processing of p100 to release RelB.

5.9 vFLIP induces κB-binding complexes composed largely of p50

EMSA supershifts were performed to investigate whether the vFLIP-induced nuclear RelB and p52 detected by immunoblot corresponded with an increase in κB binding complexes containing RelB and p52. However, Fig. 5.8 revealed a predominance of p50-containing complexes in both BC3 and Jurkat 3T8 cells expressing vFLIP. This is consistent with earlier reports that p50 is the principal NF-κB DNA binding activity induced by KSHV infection and vFLIP expression (Keller et al, 2000; Liu et al, 2002). No supershift was observed on addition of antibodies to RelB or p52 to the EMSA, although low levels of p52 and RelB were found within the nucleus. It is not clear whether this is due to the inhibitory effect of p100 found in the nucleus, to insufficient levels of RelB and p52, or to the limitations of the EMSA assay in detecting p52 and RelB dimers with the same efficiency as p50 dimers.

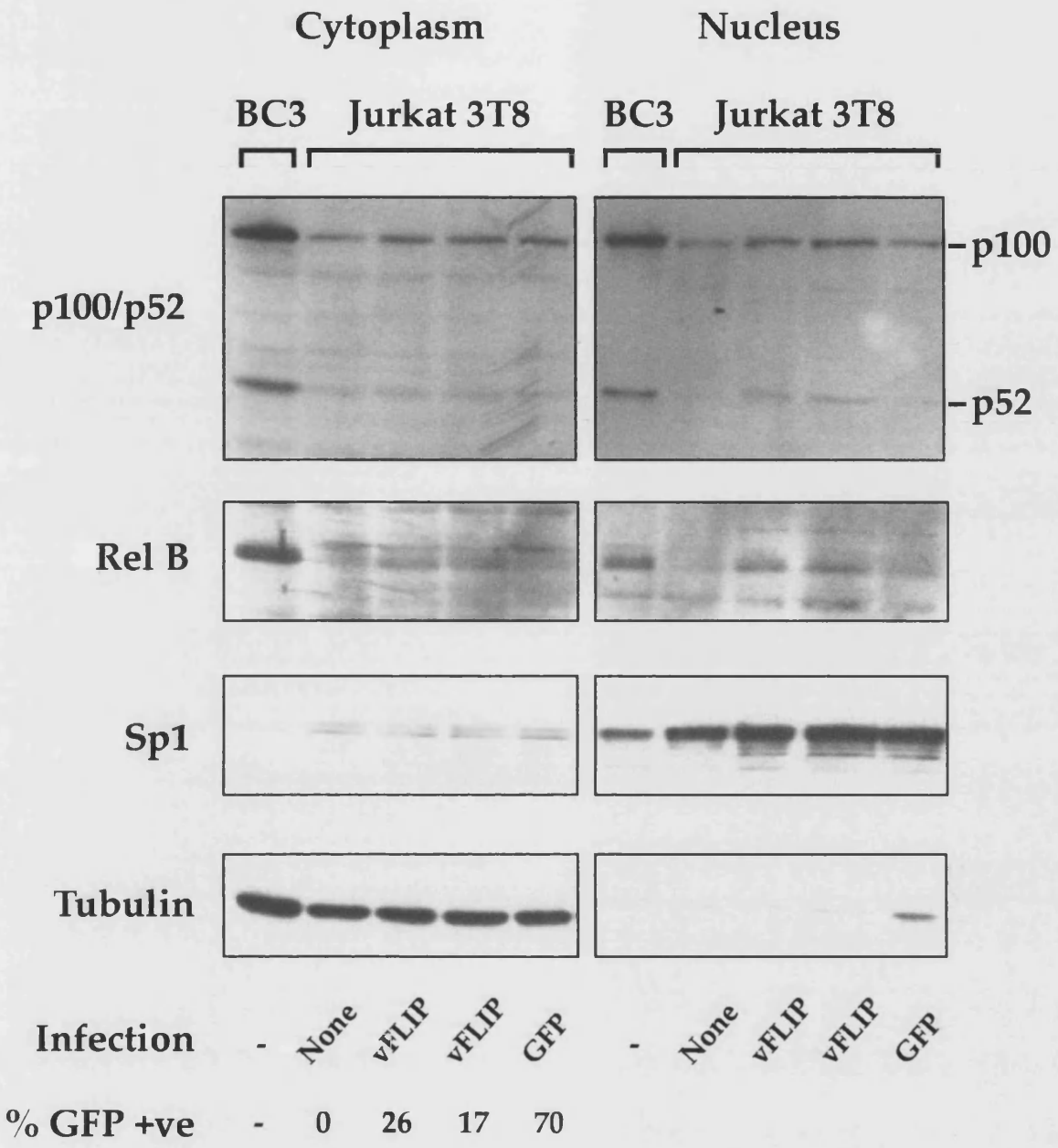


Figure 5.7. vFLIP expression induces nuclear translocation of p52 and RelB.

Nuclear and cytoplasmic extracts were prepared from $5x10^6$ BC3 and Jurkat 3T8 cells not infected or infected with lentivirus encoding either GFP or vFLIP plus GFP. 20µg of cytoplasmic extract (2%) and 30µg of nuclear extract (10%) were analysed by immunoblotting using the monoclonal anti-p100/p52, anti-RelB, anti-Sp1 and anti-tubulin antibodies. Transduction efficiency was measured by FACScan analysis of cells expressing GFP.

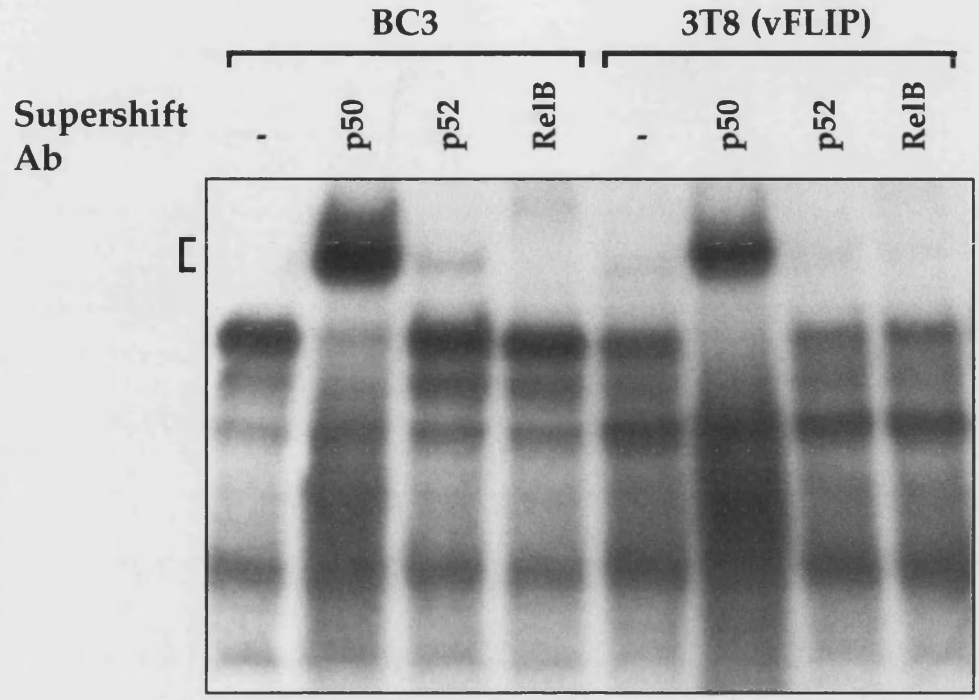


Figure 5.8. The protein/DNA complexes induced by vFLIP expression that bind κB response elements are largely composed of p50.

EMSAs were performed using total nuclear extracts (5μg) prepared from BC3 and from Jurkat 3T8 cells infected with lentivirus encoding vFLIP plus GFP, either with (as indicated) or without (-) supershifting antibodies recognising complexes containing p50, p52 or RelB proteins. The position of the supershifted complexes is shown (bracket).

5.10 Discussion

The processing of p100 and generation of p52 are essential to the development of the immune system. B cell maturation and the formation of secondary lymphoid structures do not occur in the absence of this pathway (Franzoso et al, 1998; Caamano et al, 1998), and a number of cellular receptors that stimulate this process have now been identified (Hacker et al, 2002). Two viruses, EBV and HTLV-1, encode proteins that modulate this pathway (Xiao et al, 2001a; Saito et al, 2003), and it is tempting to speculate that this has a specific role in allowing the viruses to regulate differentiation of infected cells to their own advantage. In this chapter, data have been presented suggesting that via vFLIP, KSHV also activates the alternative pathway of NF-κB activation. Co-expression of vFLIP and p100 in 293T cells and expression of vFLIP in Jurkat 3T8 and 8321 cells led to p52 accumulation. However, it remains possible that p52 accumulates as a direct result of increased p100 expression via vFLIP activation of the classical pathway, rather than through a true processing event. In support of the argument for processing is the fact that expression of exogenous p100 (driven by a CMV promoter) could not be influenced by vFLIP activation of the classical NF-κB pathway, and although it remains possible that upregulation of endogenous p100 imitated the effect of overexpression (Fig. 5.1), endogenous p100 remained below the limit of detection in these experiments. In addition, vFLIP-induced p52 generation was dependent on serine residues S866 and S870 of p100. These residues are required for processing of p100 by NIK, CD40 and Tax (Xiao et al, 2001a; Xiao et al, 2001b; Coope et al, 2002), and these data are therefore consistent with the model that vFLIP can induce the processing of p100 to generate p52.

The physical association between vFLIP and p100 provides a possible mechanism for these observations, although further experiments will help to support these data. For example, it will be interesting to define the domain of interaction required for p100 to interact with vFLIP, and it will also be important to know whether IKKγ is essential for the apparent processing of p100. To this end, experiments were performed in the IKKγ-deficient Jurkat 8321 cell line. However, this cell line was found to express a truncated IKKγ (Fig. 4.7) and to have normal NF-κB activation in response to vFLIP

expression (Figs. 4.8; 4.9). Experiments were also performed in MEF cell lines derived from genetically modified mice deficient in IKKα, β and γ, and in the murine pre-B-cell line 70Z/3 and its IKKγ-deficient mutant 1.3E2. However, neither p100 upregulation nor p52 accumulation could be demonstrated in either the wild-type or mutant murine cells (data not shown). Since p100 expression and p52 generation was observed in Jurkat cells, and p52 accumulation could be demonstrated in 293T cells, the activation of p100 processing by vFLIP may depend on its expression in a human cell line.

Although analysis of nuclear and cytoplasmic fractions revealed an increase in nuclear RelB and p52 in Jurkat cells expressing vFLIP, the levels detected by immunoblot were low. Re-infecting these cells with lentivirus to achieve greater cell numbers expressing vFLIP may help to clarify this result, but it will also be essential to perform pulse-chase experiments to define whether p100 is truly processed in cells containing KSHV and vFLIP. The failure to observe κB binding complexes containing RelB or p52 is perhaps not surprising given their low expression levels. This result may also reflect limitations of the EMSA as an assay. EMSAs are not quantitative and the intensity of bands does not necessarily correlate with amount of nuclear protein. In addition, different NF-κB dimers bind the κB consensus element with different affinity, and p52 homodimers in particular bind more weakly than many other NF-κB subunits to the commonly used consensus sequences (Fujita et al, 1992; Duckett et al, 1993).

Important questions regarding vFLIP as an activator of p100 processing still remain. The alternative pathway of NF-κB activation, where p100 processing leads to the release of RelB- and p52-containing complexes, is distinct in a number of ways from the classical pathway, that degrades small IκB molecules to release RelA:p50 dimers (Muller et al, 2003; Saccani et al, 2003). Time course experiments demonstrate that RelB-p52 complexes appear at later time points following an NF-κB activating stimulus (Muller et al, 2003; Saccani et al, 2003), and the two pathways can be examined independently by using cells deficient in the non-redundant components of each, by using inhibitors of protein synthesis that only block the alternative pathway and by using the IκB super-repressor that specifically inhibits the classical pathway.

Such experiments will prove invaluable in consolidating the existing data on vFLIP, and may help to explain how vFLIP is able to trigger the constitutive activation NF-κB.

Activation of the alternative pathway may also have an important role in viral oncogenesis. In fact, p100 was initially identified as Lyt-10, a gene involved in the B cell lymphoma-associated translocation, t(10;14)(q24;q32) (Neri et al, 1991). Such translocations have been shown to cause C-terminal truncation of p100 (Section 5.1). C-terminal truncation mutants lost their IκB-like activity and function as constitutive transcriptional activators (Chang et al, 1995). Unlike p52 and p100, overexpression of such mutants in MEFs led to a transformed phenotype (Ciana et al, 1997). Interestingly, the p52 co-activator protein Bcl-3 (Section 1.3.2), was also identified though study of chromosome translocations in chronic lymphocytic leukaemia (Perkins 2003). Since the overexpression of p100 prevented the transformation of rat embryo fibroblasts by Tax (Yamaoka et al, 1996), it would be interesting to see whether p100 also blocks transformation of Rat-1 cells by vFLIP (Sun et al, 2003b). The significance of p100 processing in survival and proliferation of KSHV-infected cells might also be tested by introducing wild-type and processing-resistant forms of p100 into PEL cell lines. Finally, it will of interest to determine whether Bcl-3 has a role in the vFLIP-induced NF-κB activation.

Summary of Results

Chapter 3.

- vFLIP is constitutively associated with an activated IKK complex, containing IKKα, β and γ and Hsp90, in KSHV-infected BC3 cells.
- vFLIP can be depleted from BC3 extract by immunoprecipitation using IKKγ antibody, suggesting this interaction is the primary function of vFLIP.
- vFLIP persistently activates IKK in heterologous cells (confirming data published by Chaudhary et al), implying that vFLIP alone can activate NF-κB.
- vFLIP-induced IKK activity can be inhibited by GA, suggesting that the activity of this complex depends on Hsp90.
- GA causes cell death in BC3 cells.

Chapter 4.

- vFLIP binds directly to IKKγ, the minimum IKKγ domain required for this interaction being amino acids 150-272.
- An IKKγ mutant consisting of amino acids 150-272 inhibits vFLIP-induced NF-κB activity in reporter-based assays.
- IKKγ truncation mutants containing the minimum domain of interaction compete with endogenous IKKγ for binding to vFLIP.
- vFLIP activates NF-κB in Jurkat cells expressing an IKKγ truncation mutant that are refractory to other stimuli.

Chapter 5.

- vFLIP stimulates p52 accumulation in transfected 293T cells, implying that the alternative pathway of NF-κB is activated.
- vFLIP-induced p52 accumulation requires p100 serines 866 and 877, suggesting that this process is phosphorylation dependent.

- vFLIP associates with p100, suggesting a possible mechanism for p52 accumulation.
- vFLIP induces both p100 and p52 accumulation in Jurkat cells, suggesting that both classical and alternative pathways of NF-κB are activated.
- vFLIP promotes nuclear translocation of p52 and RelB in Jurkat cells, suggesting that p100 is specifically processed.

Chapter 6

Discussion and future directions

6.1 Function of vFLIP

The work presented in this thesis is based upon the observation that vFLIP associated with and activated IKK in a transformed PEL cell line. The data suggest that activation of NF-κB via IKK is the major function of vFLIP in KSHV infection. Further analysis showed that IKK activation is mediated via IKKγ, and that vFLIP activates the alternative pathway of NF-κB. However, it remains possible that the observations made by Belanger et al, demonstrating the interaction between vFLIP and procaspase-8 (Belanger et al, 2001), do occur *in vivo* at certain points in the virus life-cycle. In partial support of these data is the finding that PEL express TNFR1 and Fas, but are resistant to TNF- and Fas-mediated apoptosis, although vFLIP could not be found at the DISC (Low and Collins, unpublished). Furthermore, vFLIP protected B lymphoma cells from Fas-mediated apoptosis by inhibiting caspase activation. vFLIP also permitted clonal outgrowth of these cells in the presence of Fas ligand *in vitro*, and enhanced tumour growth *in vivo* (Djerbi et al, 1999). The question therefore remains as to whether vFLIP primarily activates IKK or inhibits caspase-8, and two possible explanations are proposed to reconcile these results. First, contact with the DISC occurs through stimulus-dependent translocation of vFLIP from IKK to the DISC. Second, the interaction with caspase-8 is an artefact of overexpression, and the NF-κB-dependent expression of anti-apoptotic genes like cFLIP is responsible for the inhibition of apoptosis (Sections 6.3;6.4). The data presented here and from the published studies of Chaudhary and colleagues (Section 1.4.7) support the second explanation. However, given the precedent set by other FLIP proteins that do interact with the DISC, it will be difficult to prove that the function of vFLIP *in vivo* is exclusively related to the activation of NF-κB. This chapter explores some of the other questions concerning the function of vFLIP that remain unanswered.

6.2 How does vFLIP activate NF-κB?

Although I have shown that vFLIP contacts IKKγ directly, it is not clear how this leads to IKK activation. The most likely mechanisms for activation of IKK involve either the recruitment of upstream activators or the transautophopshorylation of the kinase subunits (Section 1.3.3.4). On the basis of several assumptions (Section 4.8), I speculated that vFLIP is able to activate the IKK complex in the absence of the IKKγ C-terminus. Previous investigators have suggested that the C-terminus is required for recruitment of upstream activators. If this speculation is correct, vFLIP is the only stimulus capable of activating IKK in absence of the IKKγ C-terminus. From this, it may be inferred that vFLIP does not require upstream activators to induce IKK activity. In this case, activation might occur through changes in the conformation of the IKK complex caused directly by the binding of vFLIP to IKK. An alternative explanation is that vFLIP itself recruits an upstream regulator, thereby circumventing the requirement for the C-terminus. However, no evidence of vFLIP binding to potential regulatory proteins was found in either mass spectrometry or yeast-two-hydrid analyses. Furthermore, Matta et al, have shown that one of the more likely candidates, RIP1, is dispensable for vFLIP-induced activation of IKK (Matta et al, 2003). It is hoped to gain a better understanding of the vFLIP-IKKγ interaction through analysis of the crystal structure of vFLIP-IKKγ complexes. A second, closely related question concerns the ability of vFLIP to induce a persistent state of activation within the IKK complex, a property shared by Tax. It was recently reported that Tax interacts with serine/threonine protein phosphatase 2A (PP2A) (Fu et al, 2003). *In vitro* studies suggest that Tax inhibits the ability of PP2A to de-phosphorylate IKK, thereby contributing to a state of persistent IKK activity. In view of other NF-κB-related properties shared by these viral proteins, it would be interesting to know whether vFLIP possesses a similar function.

6.3 Why does KSHV express vFLIP to activate NF-κB?

Many viruses employ strategies to mimic cellular activation signals and subvert intracellular signalling to their own advantage (for review see (Santoro et al, 2003) and (Hiscott et al, 2001)). Perhaps the most relevant example to KSHV is EBV, which

encodes LMP1 to mimic CD40 signalling, thereby rescuing infected B cells from apoptosis and driving their proliferation (Thorley-Lawson 2001). By expression of genes like LMP1, EBV seems capable of driving the differentiation of naïve B cells towards a memory B cell phenotype. Latently infected memory B cells thus form a reservoir for latent virus, allowing EBV to persist for the lifetime of the host. The precursor cell type for KSHV infection is not yet known, and the endpoint of infected B cell differentiation is probably the post-germinal centre plasmablast (Jenner et al, 2003). However, it seems likely that similar principles apply to KSHV, whereby the virus imitates critical cellular signalling pathways during the development of the infected cell that steer differentiation towards a long-lived B cell phenotype. Implicit within this concept is sophisticated viral gene expression, which would enable the virus to deliver the appropriate survival signal at the correct moment. Activation of NF-κB is probably one of a number of strategies that KSHV uses to drive cell proliferation, survival and differentiation of infected cells. Although the precise role of NF-κB in B cell differentiation is not yet clear, genetic experiments in mice do suggest a fundamental role in B cell maturation. Parsparakis et al, demonstrated the absence of mature B cells in mice with B-lineage-specific disruption of IKKγ and IKKβ loci (Pasparakis et al, 2002). Likewise, Kaisho et al, demonstrated a specific loss of mature B cells in lethally irradiated mice reconstituted with $IKK\alpha^{-/-}$ stem cells (Kaisho et al, 2001). This phenotype was partially reversed by transgene expression of Bcl-2, suggesting that IKKα mediates expression of genes required for the survival and development of B cells. *In vitro* data are also persuasive, Kim et al, demonstrated the essential nature of IKKγ in the survival of B cells derived from embryonic stem cells that were induced to differentiate in the presence of bone marrow cells *in vitro* (Kim et al, 2003). Knockout studies described in Section 1.3.4 have also defined a crucial role for the alternative pathway in secondary lymphoid organogenesis and B cell maturation. It is therefore tempting to speculate that by expressing vFLIP, KSHV is able to tap into NF-κB-dependent gene expression programmes and influence the fate of the infected cell.

However, these studies highlight an essential limitation of the work presented in this thesis. *In vivo*, EBV can express three different programmes of latent gene expression that seem tailored towards the differentiation state and location of the infected cell

(Thorley-Lawson 2001). The process of KSHV-driven differentiation must also be dynamic, but the work presented here concentrates on the role of vFLIP in the context of a transformed PEL cell line in culture. It would therefore be interesting to express vFLIP in primary B cells, endothelial cells and their precursors. Such experiments might include *in vitro* models of lymphoid cell development (as above) to ascertain the effect of vFLIP on differentiation and maturation in these cells. Furthermore, it is conceivable that vFLIP activates a very specific selection of NF-κB responsive genes (perhaps avoiding those involved in the immune response); microarray experiments would help to pinpoint the genes that respond to vFLIP transactivation. Indeed, through the activation of NF-κB-dependent genes, vFLIP might be argued to protect cells from apoptosis, to increase their rate of proliferation and to alter their state of differentiation (see below). The balance of gene expression may also change according to stage of infection, cell type and differentiation. Therefore, understanding the profile of NF-κB target genes would provide further clues as to the precise role of vFLIP.

An interesting feature of vFLIP activation of NF-κB is the number of "pirated" genes encoded by KSHV whose cellular homologue is regulated by NF-κB. cFLIP is a good example (Micheau et al, 2001; Kreuz et al, 2001). Other such genes that *may* be regulated by vFLIP, via NF-κB, include cyclin D1 (Guttridge et al, 1999; Hinz et al, 1999), cIL-6 (An et al, 2003b), cIAP (Wang et al, 1998) and Bcl-2 homologues, A1 and Bcl-X_L (Karin et al, 2002b). Interestingly, the corresponding viral proteins, vFLIP, vcyclin, vIL-6, vIAP and vBcl-2 respectively, all possess a range of functions that overlaps with, but often exceeds those of their cellular homologues (Sections 1.2.7; 1.2.8). It seems logical that the virus should avoid duplication of gene function, and up-regulation of the cellular genes via vFLIP may have provided evolutionary space for the viral homologues to develop novel functions. For example, if vFLIP does transactivate the expression of anti-apoptotic genes via NF-κB, the ability to bind caspase-8 and prevent DR-mediated apoptosis would seem functionally redundant. These observations may support the argument that vFLIP is solely an activator of NF-κB and does not participate at the DISC.

NF-κB and, by implication, vFLIP may also play a role in regulating viral gene expression. For example, the HSV-1 genome was found to contain κB consensus sites (Rong et al, 1992), and NF-κB has been shown to enhance viral replication through activation of viral gene expression (Patel et al, 1998; Amici et al, 2001). Conversely, NF-κB was recently suggested to maintain viral latency through repression of viral gene expression (Brown et al, 2003). In γ-herpesviruses, lytic replication involves a cascade of viral gene expression, comprising the sequential activation of immediate early, early and late genes (West et al, 2003). In KSHV, ORF50 encodes the viral regulator of transcriptional activation (RTA). RTA is sufficient to initiate the lytic phase and viral replication (Lukac et al, 1998; Sun et al, 1998), and is considered to be a key mediator of the switch from latent to lytic programmes of gene expression. It is therefore interesting that overexpression of RelA inhibited replication of MHV68 and prevented activation of the lytic promoters belonging to MHV68, EBV and KSHV (Brown et al, 2003). Furthermore, inhibition of NF-κB using Bay11-7082 (Section 1.4.7) led to the expression of proteins identical to those induced by TPA in KSHV-infected cells. These data suggest that NF-κB represses viral entry into lytic phase, and support previous observations showing that transfection of RelA antagonised the ability of RTA to transactivate some promoters, including the cIL-6 promoter (Roan et al, 2002). However, both studies are limited by the use of RelA alone to mimic NF-κB activity. It would be interesting to know whether vFLIP, as an activator of NF-κB in KSHV infection, can prevent lytic promoter activation and antagonise RTA. It would also be of interest to know whether other viral genes are NF-κB responsive.

6.4 Does vFLIP participate in KSHV-mediated cell transformation?

Although KSHV encodes a number of genes with oncogenic properties, only vcyclin, LANA and Kaposin A are expressed in latency (Table 1.2). Recent data described by Chaudhary and colleagues, and in this thesis make a strong argument that vFLIP may also have a role in the transforming ability of KSHV. Its latent expression has been demonstrated in PEL cell lines by immunoblot (Low et al, 2001), and in KS spindle cells by ISH analysis (Sturzl et al, 1999), it transformed cells *in vitro* (Sun et al, 2003b) and it induced aberrant NF-κB activity (Chaudhary et al, 1999). Furthermore,

RNAi targeting of vFLIP in BC3 cells lead to their apoptosis, although the expression of vcyclin was also reduced (Guasparri and Cesarman, unpublished). However, some data are inconsistent with vFLIP as a transforming factor for KSHV. Thus, HSV-FLIP was found to be dispensable in HVS transformation of human and simian T cells *in vitro*, and in HVS induction of T cell lymphomas *in vivo* (Glykofrydes et al, 2000). By analogy, these observations suggest that vFLIP is not required for KSHV transformation. However, direct comparison between the two viral proteins is not valid for a number of reasons. First, vFLIP is latent (Dittmer et al, 1998; Sarid et al, 1998; Sturzl et al, 1999; Jenner et al, 2001; Low et al, 2001) while HVS-FLIP (like HVS-cyclin) is expressed in late lytic phase (Thome et al, 1997), implying different roles in the viral life cycle and biology. Second, HVS expresses two latent proteins not encoded by KSHV, Tip and StpC (Neipel et al, 1998), that activate NF-κB and are both essential and sufficient for transformation (Yoon et al, 1997; Lee et al, 1999; Merlo et al, 2001). Third, the infected cells in KSHV-associated diseases are not T cells and the local environment of viral and cellular factors is therefore likely to differ. Fourth, transformation by vFLIP seems to depend upon its ability to activate NF-κB, and there is no evidence that HVS-FLIP activates NF-κB.

NF-κB has been found to have an important role in the development of certain cancers. The fact that vFLIP activates NF-κB therefore supports the concept that vFLIP has a role in KSHV-mediated transformation. Interestingly, Chaudhary and colleagues found NF-κB activity to be essential for vFLIP-mediated cell transformation (Sun et al, 2003b). It is therefore relevant that constitutive activation of NF-κB is found in a variety of lymphoid malignancies (Gilmore et al, 1996; Mosialos 1997; Karin et al, 2002a), and that NF-κB has also been implicated in the pathogenesis of solid tumours (Karin et al, 2002a). For example, there is good evidence that constitutive NF-κB activity is an important event in the development of breast cancer (Karin et al, 2002a). There are three classes of genes regulated by NF-κB that might contribute to tumourigenesis in KSHV infection: those stimulating cell proliferation, those inhibiting apoptosis and those that promote angiogenesis and metastasis (Karin et al, 2002a). NF-κB controls proliferation through regulated expression of growth factors such as IL-2 (Hoyos et al, 1989; Lai et al, 1995) and IL-6 (Libermann et al, 1990; Shimizu et al, 1990). This may be important in all KSHV-

associated neoplasms, where paracrine and autocrine activation of cell proliferation are thought to be essential (Sections 1.2.5; 1.2.8.4). Cyclin D may also be an NF-κB target (see above), contributing to the influence that NF-κB exerts on the cell cycle. NF-κB also regulates the expression of a variety of anti-apoptotic genes and can inhibit apoptosis induced by a number of different stimuli (Section 1.4.7). In the context of KSHV infection, this may allow immune escape of infected cells, and also prevent the death of aberrantly proliferating cells that would otherwise be eliminated by checkpoints on cell division (Evan et al, 2001; Karin et al, 2002a). Angiogenesis is a particular characteristic of KS (Section 1.2.4.1); it is therefore interesting that transfection of the IκBα-super-repressor into human ovarian cell lines blocked production of IL-8 and VEGF (both angiogenic chemokines) and suppressed angiogenesis *in vivo* (Huang et al, 2000). Finally, κB binding sites have been identified in the promoters of several genes that promote metastasis such as matrix metalloproteinases and chemokines (Pahl 1999; Karin et al, 2002a). In summary, NF-κB regulates the expression of a variety of genes that are potentially involved in oncogenesis. If KSHV-associated oncogenesis were dependent upon the expression of such genes, NF-κB inhibitors may provide a useful therapeutic intervention for KSHV-associated disease.

When considering the role of vFLIP in KSHV-mediated oncogenesis, the two most relevant parallels are Tax and LMP1. Both viral proteins stimulate NF-κB activation via alternative and classical pathways. Both are fundamental to viral transformation of infected cells, and there is strong evidence to suggest that cell transformation is dependent upon their ability to activate NF-κB (Section 1.3.4.4). vFLIP shares many characteristics with Tax and LMP1, and this strengthens the argument that vFLIP may represent a novel oncogenic gene responsible for KSHV-mediated transformation. However, it is striking that the expression of vFLIP is so closely linked to that of vcyclin and LANA. These three adjacent genes share the same promoter, and vcyclin and vFLIP are expressed from a single bicistronic message (Section 1.2.7.1). LANA in particular has numerous roles in regulating transcription (Section 1.2.7.2), and it has already been shown that LANA and vFLIP co-operate in inducing the expression of IL-6 (An et al, 2003b). It seems likely that vFLIP, vcyclin and LANA may work in

concert to control the infected cell, and so it follows that all three may be required for KSHV oncogenesis.

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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 as two differently spliced, polycistronic mRNAs; *LT1* is translated to produce LANA and *LT2* produces both vCyclin and vFLIP using an internal ribosome entry site (Grundhoff et al., 2001; Low et al., 2001; Renne et al., 2001; Talbot et al., 1999). vCyclin forms a complex with cyclin-dependent kinase 6 (CDK6) that is resistant to inhibition by CDK inhibitors (Chang et al., 1996; Godden-Kent et al., 1997; Swanton et al., 1997). LANA is responsible for maintaining the viral episome and interacts with p53 and pRb to interfere with their activity (Ballestas et al., 1999; Cotter et al., 1999; Friborg et al., 1999; Radkov et al., 2000).

Two roles have been proposed for vFLIP. By analogy with FLIP proteins expressed by herpesvirus saimiri, equine herpesvirus and molluscum contagiosum poxvirus, it has been suggested that vFLIP blocks Fas-mediated apoptosis (Bertin et al., 1997; Hu et al., 1997; Thome et al., 1997). Indeed, vFLIP inhibits procaspase-8 cleavage after Fas triggering (Belanger et al., 2001) and is able to promote tumour growth when expressed in a Fas-sensitive B cell lymphoma cell line (Djerbi et al., 1999). More recently, vFLIP protein has been implicated in the activation of the transcription factor NF-κB. vFLIP can activate NF-κB-driven reporter constructs in 293T cells (Chaudhary et al., 1999), and also interacts with and activates the central kinase of the NF-κB signalling pathway, IκB kinase (IKK) when ectopically expressed in a non-small-cell lung carcinoma cell line (Liu et al., 2002).

Many signals for NF-κB activation converge on the cytokine-inducible protein kinase complex IKK. The complex contains two catalytic components, IKKα and IKKβ (also called IKK1 and IKK2) (DiDonato et al., 1997; Mercurio et al., 1997; Zandi et al., 1997; Regnier et al., 1997), and a regulatory subunit, IKKγ (Rothwarf et al., 1998) [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 IκBα 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 IκBα (S32A/S36A) plasmids were generous gifts from N. Perkins (Dundee, UK). Glutathione-*S*-transferase (GST)-IκBα fusion proteins containing the first N-terminal 54 amino acids of wild-type and mutant IκBα 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, pHR′-CMV-eGFP contains a cytomegalovirus (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 *Xho*I site of pHRCMV-LacZ to produce pHRCMV-LacZ-IRES-eGFP. The *LacZ* gene was then replaced with vFLIP. IKKγ 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% CO_2. 293T cells were maintained in Dulbecco's modified Eagle's medium with 10% FCS, penicillin and streptomycin at 37°C in 10% CO_2. 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 FACScan 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). 1×10^{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 resins 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 α-cyano-4-hydroxycinammic 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 10^7 independent yeast colonies were collected, pooled and stored at –80°C in aliquots. The screen was performed to ensure that at least 5×10^7 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

2×10^7-2×10^8 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 Na_3V0_4, 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 Superose 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 LKB:μSeparation 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

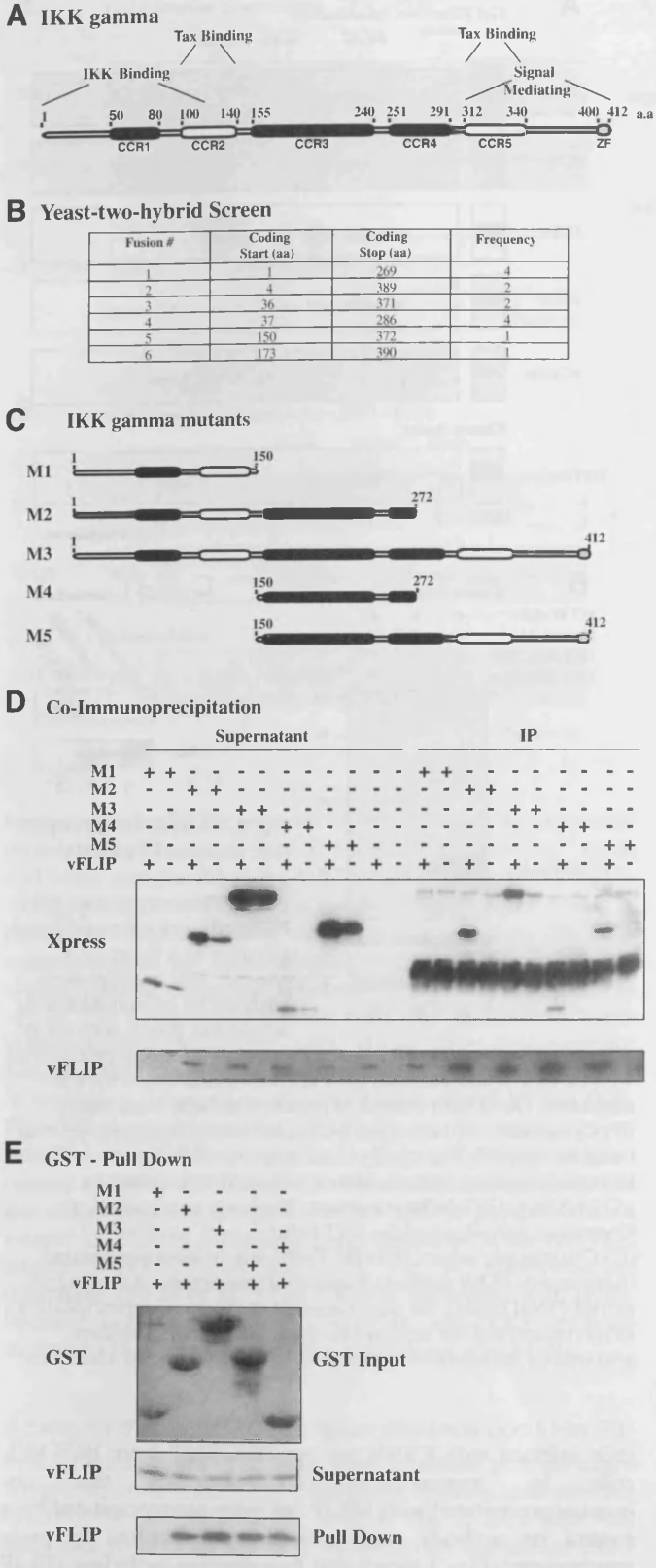


Fig. 1. vFLIP interacts directly with the IKKγ subunit of the IKK complex. A human placental cDNA library was screened for proteins interacting with a vFLIP bait using yeast-two-hybrid technology. (A) A schematic representation of IKKγ with a list of the six independent fusions of IKKγ (B). Black boxes indicate coiled-coil regions (CCR), white boxes indicate the leucine zipper domains (LZ) essential for interaction of HTLV-1 Tax with IKKγ and the grey box indicates a zinc finger motif (ZF). The N-terminus of IKKγ is responsible for interaction with IKKα and β, whereas the C-terminus is required for activation of the IKK complex. Truncation mutants of IKKγ, generated as Xpress tag or GST fusions, are shown (C). (D) The interaction of Xpress tagged IKKγ truncation mutants with vFLIP when both are overexpressed in 293T cells. Cell lysates were immunoprecipitated using an anti-vFLIP antibody and analysed by immunoblot probed with an anti-Xpress antibody. (E) Interaction of vFLIP transiently expressed in 293T cells with GST fusion IKKγ mutants.

(Sigma) or with GST-IKKγ truncation mutants pre-bound to glutathione Sepharose 4B (Amersham) for 2 hours at 4°C. The complexes were washed three times in wash buffer [20 mM Tris-HCl pH 7.5, 500 mM NaCl, 0.2% NP-40, 10% glycerol, 1 mM PMSF and 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) (Invitrogen 46-0528), anti-IKKα rabbit polyclonal (1:1000 dilution) (Cell Signalling Technology 2682), anti-IKKβ goat polyclonal (1:200 dilution) [Santa Cruz (SC)-7330], anti-IKKγ rabbit polyclonal (1:200 dilution) (SC-8330) and anti-vCyclin rat monoclonal (1:100 dilution) (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).

IκBα 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-IKKβ 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 Na_3VO_4, 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 MgCl, 2 mM DTT, 20 μM ATP, 0.1 mM Na_2VO_4 and protease inhibitor cocktail) was added. 0.5 μl of P^{32}-γ-ATP and 1 μg of wild-type IκBα_1-54 or mutant IκBα_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, 10^6 cells were washed once in cold PBS before staining with TACS™ AnnexinV-FITC Apoptosis detection kit (R&D Systems) and analysis with a FACSCaliber using CellQuest software (Becton Dickinson, Franklin Lakes, NJ).

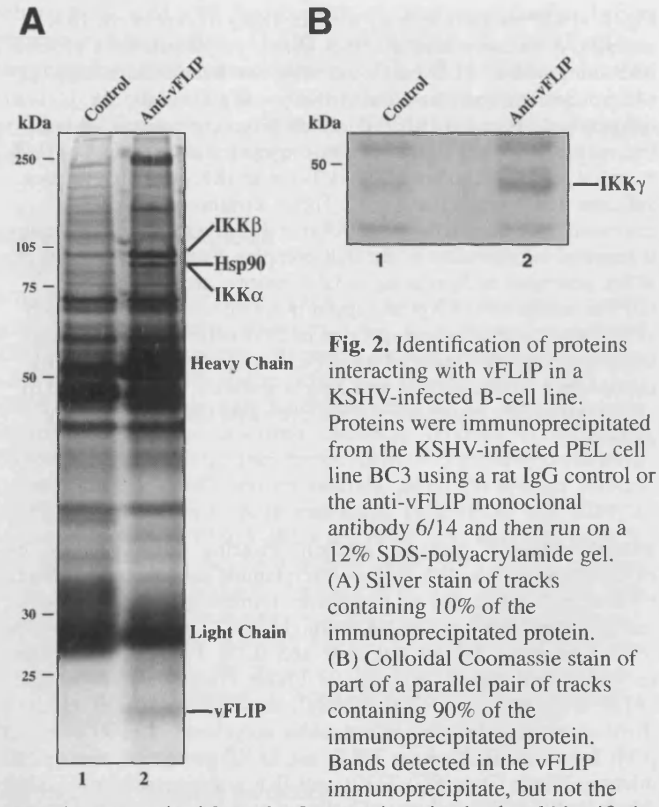


Fig. 2. Identification of proteins interacting with vFLIP in a KSHV-infected B-cell line. Proteins were immunoprecipitated from the KSHV-infected PEL cell line BC3 using a rat IgG control or the anti-vFLIP monoclonal antibody 6/14 and then run on a 12% SDS-polyacrylamide gel. (A) Silver stain of tracks containing 10% of the immunoprecipitated protein. (B) Colloidal Coomassie stain of part of a parallel pair of tracks containing 90% of the immunoprecipitated protein. Bands detected in the vFLIP immunoprecipitate, but not the control, were excised from the Coomassie stained gel and identified by mass spectrometry as indicated.

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 PEL cells by immunoprecipitation. Proteins that co-immunoprecipitated with vFLIP but were not precipitated by a control rat antibody were excised and identified by mass spectrometry. Fig. 2 shows that five proteins including vFLIP were identified in the vFLIP lane but not in the control lane. All five proteins were clear matches with high Mascot scores (Perkins et al., 1999). Three of these proteins were identified as the core components (IKKα, IKKβ and IKKγ) of IKK. The

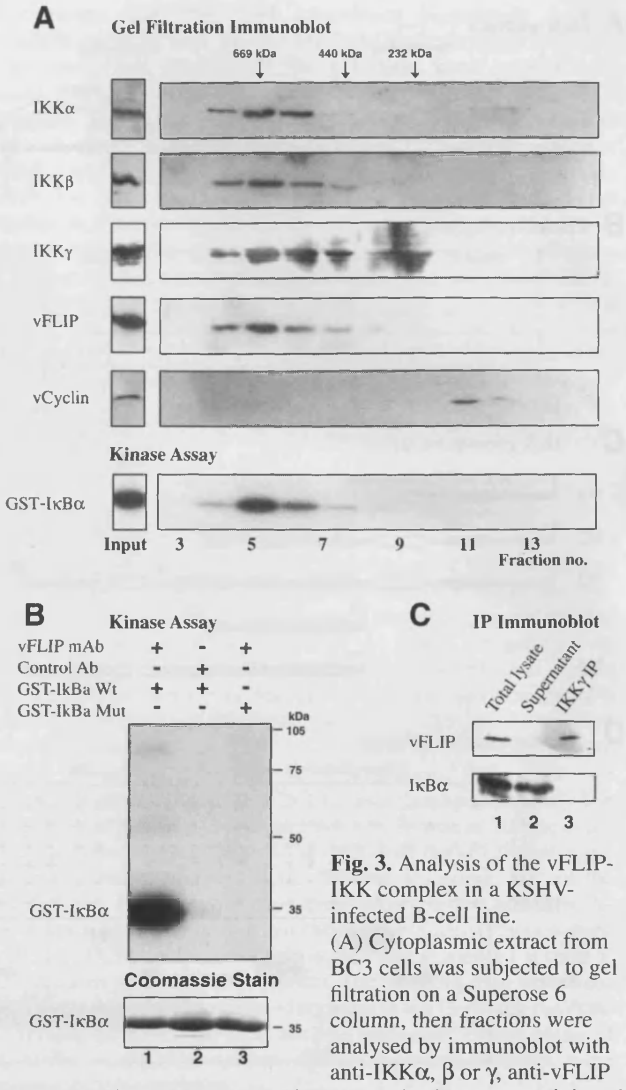
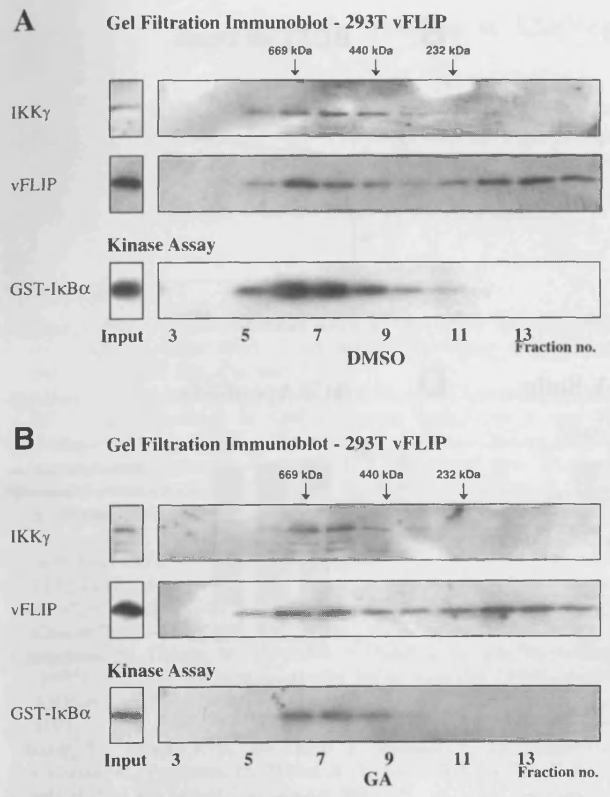


Fig. 3. Analysis of the vFLIP-IKK complex in a KSHV-infected B-cell line. (A) Cytoplasmic extract from BC3 cells was subjected to gel filtration on a Superose 6 column, then fractions were analysed by immunoblot with anti-IKKα, β or γ, anti-vFLIP and anti-vCyclin antibodies. Fractions were also immunoprecipitated with the anti-vFLIP antibody 6/14 and IκBα kinase activity was measured. The elution volume of protein standards is indicated. (B) Cytoplasmic extracts from BC3 cells were immunoprecipitated using the anti-vFLIP antibody or an isotype-matched control. The immune complexes were incubated with wild-type or mutant (S32A/S36A) GST-IκBα substrates. The lower panel shows a Coomassie stained gel of the GST-IκBα in each reaction. (C) Cytoplasmic extract from BC3 cells was immunoprecipitated using an anti-IKKγ antibody. Equivalent proportions of the original extract (Total lysate), the supernatant from the immunoprecipitation (Supernatant) and the immunoprecipitate (IKKγIP) were then analysed by immunoblot with anti-vFLIP and anti-IκBα antibodies.



band containing IKKα was also found to contain the chaperone protein, Hsp90, which has recently been identified as an additional component of the IKK complex (Chen et al., 2002).

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 (IKKα, IKKβ and IKKγ) eluted from the Superose 6 column in the same fractions as vFLIP. The Superose 6 fractions were also analysed for IκBα 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 expressing both vFLIP and GFP. Fig. 4A shows that IKKγ and activated IKK were associated with vFLIP in the transduced 293T cells. Anti-vFLIP antibody also co-immunoprecipitated IKKα and IKKγ in these cells (data not shown).

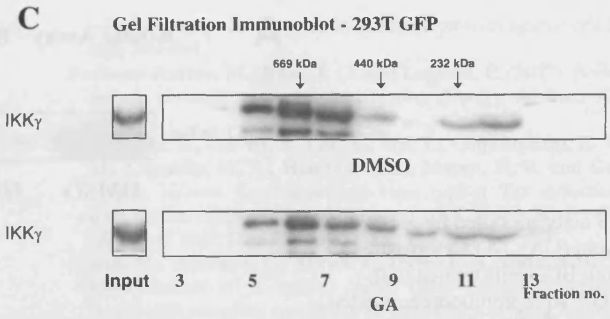


Fig. 4. The vFLIP-IKK kinase activity is inhibited by geldanamycin. Cytoplasmic extract from 293T cells transduced with a lentivirus encoding either vFLIP plus GFP (A,B) or GFP alone (C) and then treated with DMSO or geldanamycin (GA), as indicated, was subjected to gel filtration on a Superose 6 column. 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 IKKα, 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-

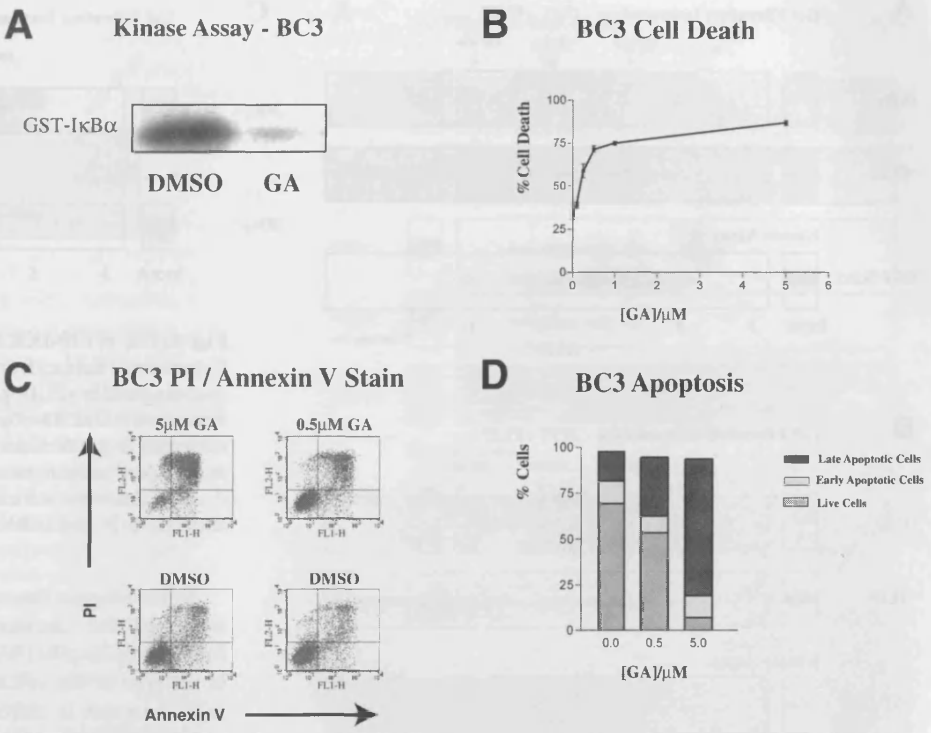


Fig. 5. BC3 cells are killed by geldanamycin (GA). (A) Cytoplasmic extracts from BC3 cells treated with DMSO or GA were immunoprecipitated using the anti-vFLIP antibody and their GST-IκBα kinase activity measured. The viability of BC3 cells treated with increasing concentrations of GA or equivalent DMSO was evaluated by haemocytometer (B) and by propidium iodide (PI)/annexin V stain followed by FACScan analysis (C) at 48 hours. In each of the four graphs in C, the lower left quadrant represents live unstained cells, the lower right quadrant represents single stained early apoptotic cells, the upper right represents double stained, late apoptotic cells, and the upper left represents necrotic cells. (D) Graphical representation of the data in C.

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 (Chiou 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 co-operative, 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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