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Translational initiation controls localization and regulatory function of the γ–herpesviral protein *kaposin*

vorgelegt von
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Erklärung

Diese Dissertation wurde im Sinne von §13 Abs. 3 bzw. 4 der Promotionsordnung vom 29. Januar 1998 von Prof. Dr. Rudolf Grosschedl betreut.

Ehrenwörtliche Versicherung

Diese Dissertation wurde selbständig, ohne unerlaubte Hilfe erarbeitet.

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Habent sua fata libelli

Terentianus Maurus

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1 Summary

Kaposi's Sarcoma Associated Herpesvirus (KSHV) or Human Herpesvirus-8 (HHV-8) is the most recently identified human γ-2 herpesvirus and has been implicated in Kaposi's Sarcoma (KS) and primary effusion lymphoma (PEL). At the right end of the genome KSHV encodes the complex *kaposin* locus, which consists of two distinct sets of 23 amino acid direct repeats, DR2 and DR1, followed by a short domain originally referred to as open reading frame (ORF) K12. Translational initiation at multiple alternative CUG and one AUG start codons causes expression of a gradient of *kaposin* molecules with varying length and targeting motifs from one single transcript.

The aim of the present study was to investigate in detail the expression pattern of the *kaposin* locus and the cellular localization and function of *kaposin* protein isoforms expressed in the KSHV+ PEL cell line BCBL-1. The multitude of translational products from all three reading frames could be resolved and different isoforms assigned to distinct cellular compartments. Depending on the alternative start codon used, the DR1 repeats representing a functional effector domain are fused either to the DR2 repeats harboring a nuclear localization sequence (NLS), or to K12, which encodes a transmembrane domain. Expression of *kaposin* in the nucleus (*kaposin B*) causes an activation of the AP-1 transcription factor and cellular promoters. The observed AP-1 induction is dependent on nuclear localization of both DR2 and DR1 repeats, since substitution of DR2 with a SV-40 NLS was not sufficient to restore activation. Other *kaposin* isoforms which are found in the cytosol (*kaposin E*) or membrane-associated (*kaposin D*) failed to activate AP-1. If co-expressed, however, *kaposin D* and *E* were able to modulate the *kaposin B*-caused induction, presumably mediated by a direct interaction between DR2 and DR1.

The results presented in this study indicate a novel autoregulatory mechanism based on bidirectional targeting of a viral protein to distinct subcellular compartments by expression from different start codons and reading frames. Supported by the complexity of the translational program and the conservation of the repeat regions, these findings imply that *kaposin* isoforms have important functions in the viral life cycle.

2 Introduction

2.1 Herpesviruses

The family of *Herpesviridae* encompasses more than 100 different species in animals and human. A typical herpesvirus virion consists of four structural components. In the center a core range is located, which contains the linear double stranded DNA. This core range is encased by an 100 to 110 nm spanning icosahedral capsid, which consists of 12 pentameric and 150 hexameric capsomers. Both, core and capsid together form the so-called nucleocapsid. The capsid is surrounded by an amorphous substance, the tegument, which consists of electron-dense material and can vary in its density; it is most probably responsible for the varying diameter of the different herpes virions (from 120 nm to nearly 300 nm). Tegument and nucleocapsid are enclosed by a membrane of cellular origin (envelope) containing virally encoded glycoproteins (spikes) (Fig. 1).

The genomes of herpesviruses differ both in size and in GC-content. The GC-content varies between 32% in canine herpesvirus and 75% in herpesvirus simiae. Varicella Zoster Virus (VZV) possesses among the so far described herpesviruses with approximately 125 kbp the smallest, the humane and the murine cytomegalovirus (HCMV and MCMV, respectively) with approximately 230 kbp the largest genome(s) with a coding capacity for approximately 200 proteins (Chee et al., 1990; Rawlinson et al., 1996) .

Although the length of the DNA is specific for each herpesvirus, the differences in genome size can vary up to 10 kbp within independent isolates of a virus species, which reflects usually a different number of terminal or internal repetitive sequences. A further peculiarity of all herpesviruses is the presence of virus-specific enzymes and other factors, which are involved in the nucleic acid synthesis (e.g. DNA polymerase, helicase, primase) and in the DNA metabolism (e.g. thymidine kinase, dUTPase). In addition, all herpesviruses encode at least one protease and a differing number of protein kinases.

Viral DNA synthesis and the assembly of the capsids take place in the nucleus of the host cell. During exit of the nucleus through the nuclear membrane capsids become enveloped. With some herpesviruses this first envelope is removed and replaced by a new membrane from cytoplasmatic organelles. A further typical characteristic of

herpesviruses is the irreversible destruction of the host cell during the production and release of infectious virus progeny. However, the probably most important and characteristic feature of all herpesvirus species is the ability to switch after an often asymptomatic primary infection into a state of latency and to persist life-long in the host. In latently infected cells, the virus genome is present extra-chromosomally and only few viral genes are expressed. Thus, during latency no infectious virions can be isolated from infected tissue. Due to endogenous and exogenous factors (e.g. stress, immunosuppression, UV-light, hormones etc.) the herpesvirus can reactivate and disease symptoms reoccur. The family of the *Herpesviridae* can be divided into the three *Alphaherpesvirinae*, *Betaherpesvirinae* and *Gammaherpesvirinae* subfamilies. The α-herpesviruses are characterized by the fact that they exhibit a broad host range and a short replication cycle. The infection spreads in cell culture fast and leads to an efficient destruction of infected cells. α-herpesviruses establish latent infections in sensory ganglia. Important representatives of human pathogenic α-herpesviruses are the Herpes Simplex Virus type 1 (HSV-1) and type 2 (HSV-2), which cause blisters in the lip and genital region, and the Varicella Zoster Virus (VZV), the causative agent of varicella (chickenpox) and Zoster (shingles). Contrary to the α-herpesviruses, the β-herpesviruses show a pronounced host specificity, a long reproduction cycle and a slow propagation in cell culture. The size of infected cells is frequently increased (cytomegalic), which was taken in account in the naming of some β-herpesviruses (e.g. HCMV, MCMV). β-herpesviruses can establish latency in different cells and tissues. The γ-herpesviruses are characterised by a restricted host specificity. Usually their host range is limited to the family from which their natural host originates. *In vitro*, all γ-herpesviruses replicate in lymphoblastoid cells and some also cause lytic infections in epitheloid cells and fibroblasts. This herpesvirus subfamily has a selectivity for either T or B lymphocytes, in which latent virus preferentially can be detected. The most well-known human representative is the B-cell-specific Epstein-Barr Virus (EBV), which is the causative agent of infectious mononucleosis (“kissing disease”). EBV is an oncogenic virus and associated with two endemic tumors, Burkitt’s lymphoma and nasopharyngeal carcinoma, as well as with Hodgkin’s disease. KSHV, another representative of the γ-herpesvirus subfamily is also associated with several tumor entities, similar to EBV (Chee et al., 1990; Roizman, 1996).

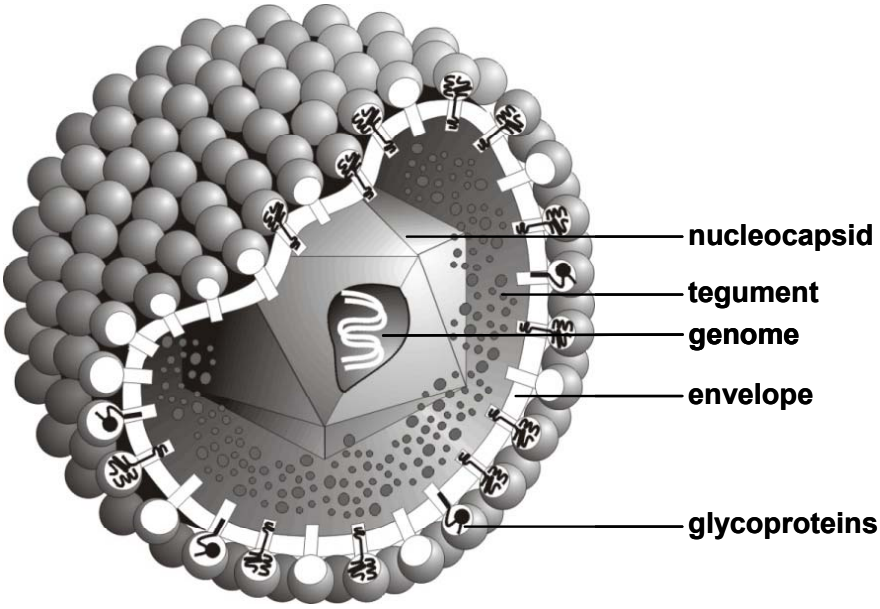


Fig. 1: The herpesvirus particle

Schematic model of a herpesvirus particle (adapted from Reschke, 1994). Major virion components are indicated.

2.2 Replication cycle of herpesviruses

The infection of a cell begins with the specific binding of virus envelope proteins to receptor molecules on the surface of the host cell. After adsorption of the virions the viral envelope fuses with the cell membrane and the nucleocapsid is released into the cytoplasm. The uncovered virus genome is circulized and transported into the nucleus, where transcription and replication take place. The replicated virus DNA is packed into capsids, which receive their first envelope by budding at the inner nuclear membrane. Depending on the herpesvirus species the first envelope membrane is replaced in the Golgi or ER and the virus progeny is released by budding.

Gene expression in herpesviruses is cascade-like regulated and can be divided in three distinct phases: *immediate early* (IE), *early* (E) and *late* (L) (Honess and Roizman, 1974). The *immediate early* phase begins immediately after the infection. For the transcription of the IE genes no *de novo* synthesis of viral proteins is necessary. IE proteins possess predominantly regulatory functions, and at least one

IE protein is necessary for the initiation of the *early* phase (Honess and Roizman, 1975). The activation of the *early* genes takes place primarily on the transcriptional level (Godowski and Knipe, 1986). During the *early* phase proteins are produced which are necessary for replication of the viral genome (e.g. viral DNA polymerase). The start of DNA replication defines the beginning of the *late* phase. In the *late* phase mainly structural proteins necessary for the formation of the virions are synthesized.

2.3 Kaposi's Sarcoma (KS)-Associated Herpesvirus (KSHV)

2.3.1 Disease association

The Hungarian dermatologist Moritz Kaposi working in Vienna was the first who described Kaposi's Sarcoma in 1872. He published a case report of five men with "*idiopathic multiple pigmented sarcoma of the skin*" including a patient who developed visceral disease in the lung and gastrointestinal tract (Antman, 2000). Two decades later this idiopathic multiple pigmented sarcoma of the skin was termed KS according to the proposal of another prominent dermatologist, Kobner, and is now referred to as classic KS. In central Africa endemic KS is one of the most frequent tumors whereas in North America and Northern Europe KS appeared rarely before the acquired immunodeficiency syndrome (AIDS) epidemic. However, the AIDS epidemic made KS to the most common AIDS-associated cancer and thus it contributes considerably to morbidity and mortality in AIDS patients (Ahmed et al., 2001). In addition, HIV seronegative, homosexual men have a higher risk for developing KS in comparison to individuals in countries where the rates of KS are higher (Ganem, 1997). KS is one of the most frequent post-transplant neoplasms predominantly after kidney transplantation. These post-transplant KS tumors regress when immunosuppressive therapy is stopped, suggesting the importance of the host immune system (Penn, 1978). KSHV is the most recently discovered human γ-herpesvirus and shows tropism primarily for endothelial cells and B-lymphocytes, but can also infect other cell types with limited efficiency. It is the eighth human herpesvirus isolated to date and is therefore also named Human Herpesvirus 8 (HHV-8) (Antman, 2000; Chang et al., 1994). KSHV was initially isolated from KS tissue but was later also found to be associated with pleural effusion lymphomas (PEL [body cavity-based lymphomas (BCBL)]) (Chang et al., 1994).

Although other pathogenic agents (among others CMV, HIV-1 and mycoplasm) were isolated from Kaposi's Sarcoma, a preponderance of data strongly suggests that KSHV is the etiologic agent of KS and may also be a critical player in the development of other lymphoproliferative disorders such as PEL and multicentric Castleman's disease (MCD) (Arvanitakis et al., 1996; Beral et al., 1990; Boshoff et al., 1995; Renne et al., 1996b; Siegal et al., 1990). Most PEL are positive for KSHV and EBV (80-90%), which is reflected by the occurrence of both viruses in cell lines derived from this tumor.

2.3.1.1 Kaposi's Sarcoma (KS)

Kaposi's Sarcoma is clinically most relevant among the KSHV associated tumors. It is an unusual neoplasm characterized by multifocal dark brown or purple lesions and differs from most other tumors by several characteristic features (Fig. 2). In KS, the lesions contain multiple cell types, of which the endothelial derived spindle cells are predominant (Boshoff et al., 1997). The clonality of KS is controversely discussed (Judde et al., 2000; Gill et al., 1998; Rabkin et al., 1997). Additionally, the KS lesions are characterized by the infiltration of inflammatory leukocytes as well as a profusion of neovascular elements (Monini et al., 1999). In immunocompetent patients KS is a slow growing tumor with low malignant potential (Ganem, 1997). In immunocompromised individuals, KS is more aggressive and can be letal. In cases where the immune competence was restored, complete remission of the disease state was observed, which is quite different from other aggressive tumors (Boshoff et al., 1997; Fiorelli et al., 1998). The presence of KSHV in PEL has been documented and coinfection with EBV was shown for the majority of cloned cell lines, including BC-1 and BC-2 (Cesarman et al., 1996). However, several PEL cell lines including BC-3 and BCBL1 were described, which showed no detectable levels of EBV (Arvanitakis et al., 1997; Renne et al., 1996b). Although B-cell markers are completely down-regulated, the clonal immunoglobulin heavy chain rearrangement indicated that these cells are of B-cell origin. KSHV is able to infect human B-cell lines and may be involved in the pathogenesis of PEL in HIV-positive AIDS patients. KSHV is also able to infect and replicate in other cell lines, but considerably less efficiently than seen in the PEL cell lines (Cerimele et al., 2001; Foreman et al., 1997). Four distinct clinical variants of KS can be distinguished. Classic KS is a

severly growing, little aggressive tumor, which typically affects elderly men of Mediterranean and eastern European origin and is mostly indolent; endemic KS, which is frequent in equatorial, eastern and southern Africa and is a clinically more aggressive form than classic KS (Wabinga et al., 1993); post-transplant or iatrogenic KS, which develops in patients undergoing immunosuppressive therapy to prevent graft rejection after organ transplantation (Regamey et al., 1998) and finally, AIDS-associated KS, the most aggressive form of the disease, is most frequently seen in gay and bisexual men, indicating that transmission is likely through high risk sexual practices (Gao et al., 1996).

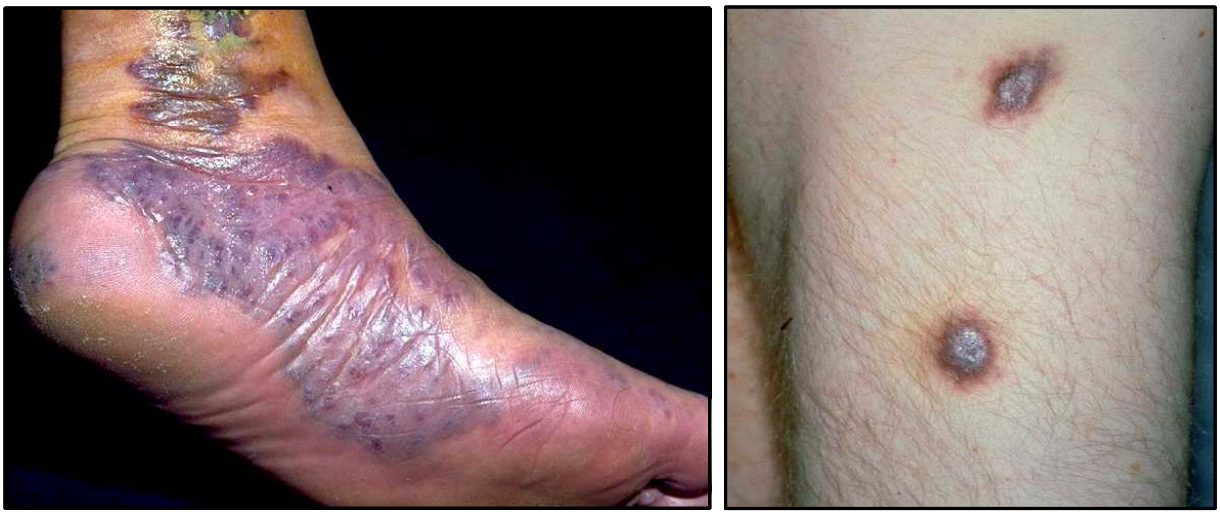


Fig. 2: Cutaneous forms of a Kaposis's Sarcoma

(A) Kaposi's Sarcoma of the lower leg and foot. Lesion at the lower leg are plaque-like, brown and sharply defined. Confluent Lesions at the foot exhibit firm purple nodes (B) AIDS-related Kaposi's Sarcoma of a 29 year-old man. Lesions are multifocal distributed in form of dark purple nodes (pictures online published in the Dermatology Online Atlas [http://www.dermis.net/doia/] according to Diepgen and Eysenbach, 1998).

2.3.1.2 Primary effusion lymphoma (PEL)

PEL (previously termed BCBL), is a rare, rapidly fatal, non-Hodgkin's malignancy associated with KSHV infection. In general, it is present as a pleural or pericardial effusion without a detectable mass or peripheral lymphoadenopathy (Arvanitakis et al., 1996). Additionally, PEL can also manifest as a solid mass in the lymph nodes, lungs or the gastrointestinal tract. PEL is found mainly in HIV seropositive individuals in advanced stages of immunosuppression, but also in HIV seronegative patients. Although EBV negative and KSHV positive PEL have been described, PEL cells are

frequently co-infected with both viruses. Southern blot analysis revealed that the copy number of KSHV genomes in PEL cells is maintained at 50-150 copies per cell, which is substantially more than the numbers observed in KSHV-infected spindle cells.

2.3.1.3 Multicentric Castleman's disease (MCD)

The multicentric Castleman's disease belongs to the atypic- or pseudo-lymphoma and is thought to be mediated by interleukin (IL)-6 overexpression (Ablashi et al., 2002). The correlation between KSHV viral load and the course of the disease suggests a functional role of KSHV in MCD (Grandadam et al., 1997).
The virus is detected in most HIV-seropositive cases of MCD as well as in approximately 40% of HIV-seronegative MCD cases. The KSHV positive MCD cases are now understood as a distinct subset of MCD, termed plasmablastic MCD, which are characterized by the occurrence of large plasmablastic cells harbouring KSHV (Dupin et al., 2000). Unlike PEL cells, co-infection by EBV has not been detected in MCD plasmablasts. The rate of lytically infected tumor cells is considerably higher in MCD in comparison to KS and PEL, suggesting a different role of KSHV in pathogenesis (Cathomas, 2000).

2.3.2 The KSHV particle

KSHV shows a typical herpesvirus morphology: virus particles have a diameter of 100- to 150-nm with a lipid envelope and an electron-dense central core (Renne et al., 1996a). The icosahedral capsid consists of 162 hexagonal capsomeres and is approximately 125 nm (1250 Å) in diameter (Wu et al., 2000). Three types of capsids, named A, B and C, are released from PEL cells after TPA and sodium butyrate treatment (Fig. 3). Fully mature C-capsids contain, in declining order of abundance, the polypeptides ORF25/MCP (major capsid protein), ORF65/SCIP (small capsomer-interacting protein), ORF26/TRI-2 (triplex-2), ORF62/TRI-1 and the 160- to 170-kb viral genome. They have a total mass of approximately 300 megadaltons. A and B capsids are constructed similarly but lack viral DNA. In addition, the B capsids contain the scaffolding protein encoded by ORF17.5 (Nealon et al., 2001). Mature

virions carry a glycoprotein coat and between the capsid and the envelope a protein-filled region, the tegument is located. The central core is torus-shaped, 75-nm in size and composed of DNA and protein. In appearance, KSHV is not distinguishable from α-, β-, and other γ-herpesvirus particles (International Agency for Research on Cancer, 1997).

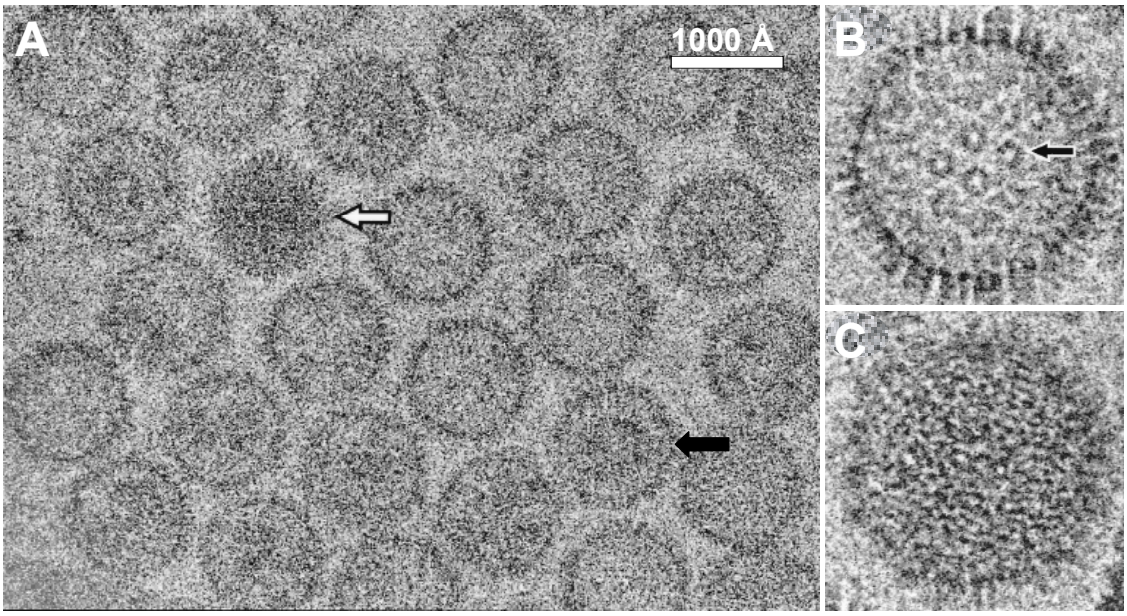


Fig. 3: Electron cryomicroscopy of HHV-8 capsids

(A) Empty A-capsids, one B-capsid (black arrow) and one DNA containing C-capsid (white arrow).(B) Enlarged view of an intermediate B capsid, which contains scaffolding protein. Characteristic hexagonal pattern of the capsomeres (e.g. arrow) is indicated. (C) Fully mature C-capsid with characteristic striated fingerprint-like pattern (adapted from Wu et al., 2000).

2.3.3 The KSHV genome

KSHV is a member of the γ2-subgroup of the γ-herpesvirus family, rhadinovirus genera, which share a collinear genomic organization with each other. The coding capacity of the KSHV genome was determined by sequencing viral DNA of a PEL cell line as well as of KS biopsy specimens, both revealing the characteristic synteny of rhadinoviruses (Russo et al., 1996; Neipel et al., 1997). Supplementing this approach, Gardella gel analyses were performed to specify the size and conformation of the viral nucleic acid (Renne et al., 1996a). During latency, the KSHV genome of PEL cell lines is maintained as a circular, multicopy episome (similar to the Herpesvirus saimiri [HVS] and EBV genomes) and includes multiple GC-rich,

801-bp terminal repeats enclosing approximately 145 kb of “unique” sequence (Lagunoff and Ganem, 1997; Moore and Chang, 2001). During the lytic replication cycle, viral progeny DNA is ultimately synthesized as linear, single-unit genomes destined for packaging into mature virions (Renne et al., 1996a).

KSHV harbors at least 89 ORFs. A comparison between KSHV and HVS (the prototype γ2-herpesvirus) reveals a strikingly similar genetic arrangement (Neipel et al., 1997; Russo et al., 1996). Both viruses share 68 conserved genes that are arranged collinearly, interrupted by interspersed regions of genes unique to each virus. All genes were numbered consecutively from the left to the right side of the genome. The conserved genes have been marked by the prefix “ORF” and the unique genes were designated K1 to K15 (Fig. 4) (Russo et al., 1996). More recently, the publication of the complete DNA sequences of the murine gammaherpesvirus 68 and several primate rhadinoviruses confirmed the conservation of this genetic organization and expanded it to non-human members of the γ2-herpesviruses family (Alexander et al., 2000; Searles et al., 1999; Virgin et al., 1997). Those genes which display the highest degree of conservation among these viruses are predicted to have metabolic and catalytic functions in replication of the viral DNA or contribute to the virion structure and are taken together in a set of “ancient” genes conserved in all mammalian herpesviruses (McGeoch and Davison, 1999; Simas and Efstathiou, 1998). In KSHV, these include the DNA polymerase and the processivity factor (ORF9 and ORF59, respectively), the DNA helicase-primase (ORF40, ORF41, and ORF44), the thymidylate synthase (ORF70), and the thymidine kinase (ORF21). Characteristically, KSHV as well as other γ-herpesviruses harbor a large number of ORFs which share homology to known cellular genes and are postulated being pirated from host chromosomes during viral evolution. Some of these genes participate in the down-modulation of the immune response, circumvent cellular systems of targeting infected cells or are involved in cell growth, differentiation and nucleotide biosynthesis. They include the Bcl-2, IL-8R, and MIP-IK, vIL-6, DHFR and the D-type viral cyclin, whose functions are usually distinct to that of their cellular homologs (Alexander et al., 2000; Russo et al., 1996). The KSHV genome also contains two lytic origins of DNA replication, that are inverted duplications of each other: the left is located between K4.2 and K5, and the right between K12 and ORF71 (AuCoin et al., 2002; Lin et al., 2003).

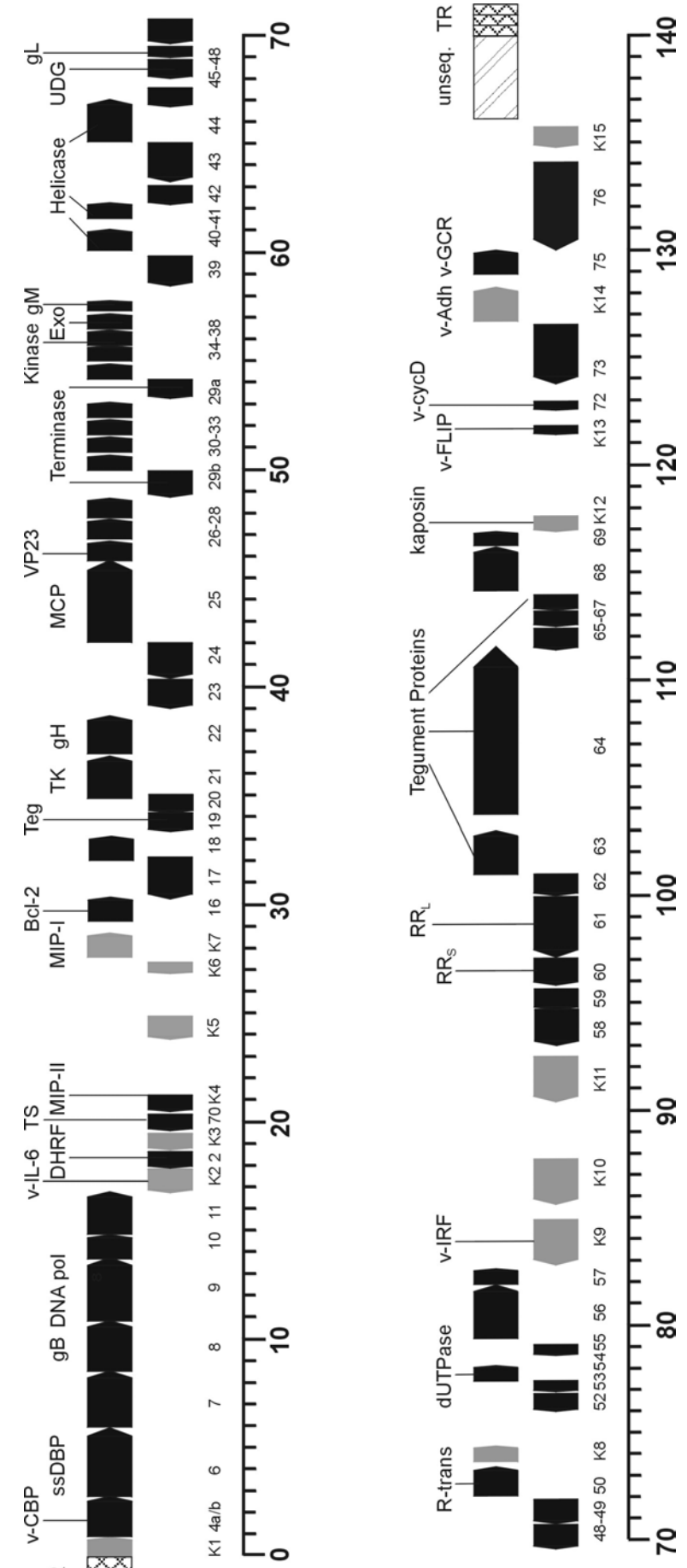


Fig. 4: Schematic map of the KSHV genome

The orientation of identified ORFs are denoted by the direction of arrows (see also text). Length is indicated in kilobases. Abbreviations: TR, terminal repeats; v-CBP, viral complement binding protein; ssDBP, single-stranded DNA binding protein; gB, gH, gM, gL, glycoprotein B,H,M,L; DNA pol, DNA polymerase; v-IL-6, viral interleukin 6; DHRF, dihydrofolate reductase; TS, thymidate synthase; MIP-I, MIP-II, macrophage inflammatory protein I, II; Teg, tegument; TK, thymidine kinase, MCP, major capsid protein; VP23, capsid protein; Exo, alkaline exonuclease; UDG, uracil DNA glucosidase; R-trans, transactivator; v-IRF, viral interferon regulatory factor; RR_S, RR_L, ribonucleotide reductase, small, large; v-FLIP, viral fas-ligand IL-1 β-converting enzyme inhibitory protein; v-cycD, viral cyclin D; v-Adh, viral adhesion molecule; v-GCR, viral G protein coupled receptor.

These genomic analyses identified the viral DNA polymerase gene as the gene with the highest intervirus identity, facilitating the construction of rhadinoviral phylogenetic trees which include KSHV, HVS, and the primate rhadinoviruses that have been identified over the last half decade (Fig. 5). The group of rhadinoviruses has since been subdivided into those of New World and Old World primates (Greensill et al., 2000b). Probably the most closest relative of KSHV is the *Pan troglodytes* (chimpanzee) rhadinovirus 1 (PtRV-1), which encodes a DNA polymerase gene that has 93.2% amino acid identity to the KSHV polymerase (Greensill et al., 2000a).

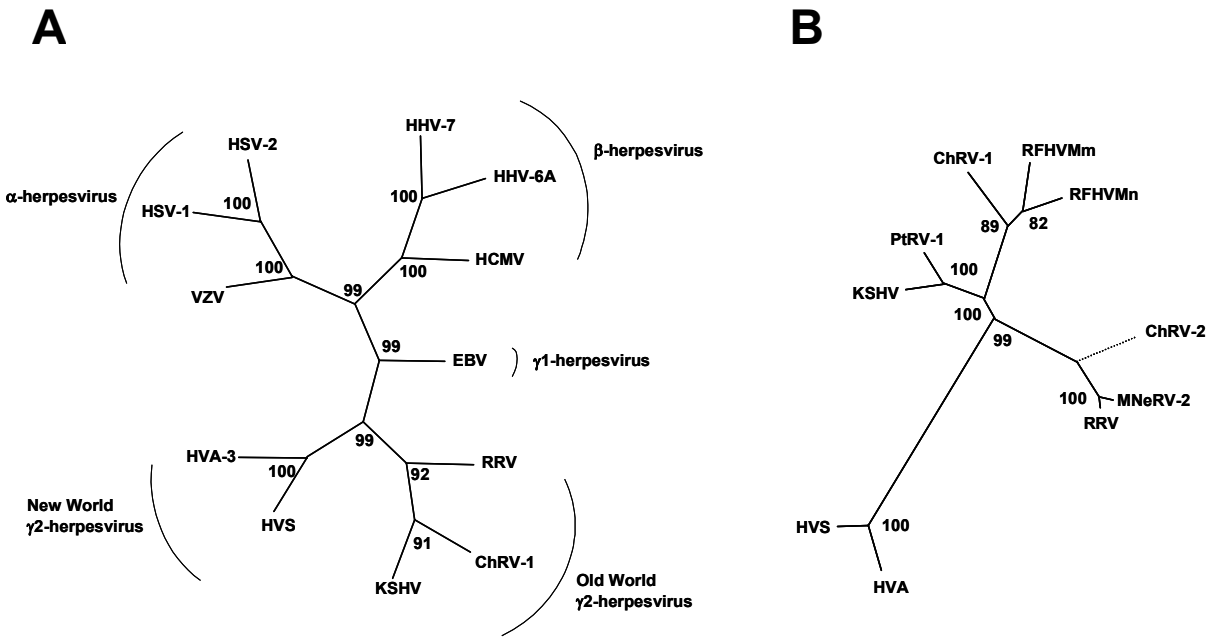


Fig. 5: Phylogenetic trees

(A) Rhadinoviruses divide into a New World- and an Old World-subgroup. DNA maximum likelihood tree for herpesviruses (Greensill et al., 2000b). (B) *Pan troglodytes* rhadinovirus 1 is the closest relative to KSHV found so far. Neighbour-joining protein distance tree of different rhadinoviruses (Greensill et al., 2000a).
Abbreviations: HSV, Herpes simplex virus; VZV, Varicella zoster virus; HHV, Human herpes virus; HCMV, Human cytomegalovirus; EBV, Epstein-Barr virus; HVA, Herpesvirus ateles; HVS, Herpesvirus saimiri; RRV, Rhesus rhadinovirus; CHRV-1,2, Chlorocebus rhadinovirus 1 and 2; RFHVMm, Mn, Retroperitoneal fibromatosis herpesvirus of rhesus and pigtailed macaques; MneRV-2, rhesus and pigtailed macaque rhadinovirus; PtRV-1, *Pan troglodytes* rhadinovirus 1. Numbers refer to the percentage of repeated analyses that gave the same tree topology ("bootstrap" values).

2.3.4 Latent and lytic gene expression in KSHV

As all herpesviruses, KSHV is able to infect cells latently (non-productive) and lytically (productive). This biphasic life cycle is characterized by a distinct gene expression program in each case and was recognized early in both KS lesions and cultured PEL specimens (Miller et al., 1996; Miller et al., 1997; Renne et al., 1996b; Staskus et al., 1997; Zhong et al., 1996). Productive infection by herpesviruses leads to cell lysis, which obviously contradicts the ability of a virus to transform the infected host cell. Thus, assigning the expression of individual KSHV ORFs to the latent or lytic cycle is decisive for predicting their potential roles in the pathogenesis of the disease. This was markedly facilitated by the ease of culturing PEL cells latently infected with KSHV, and inducing lytic reactivation with common laboratory chemicals (such as phorbol esters or sodium butyrate). If the cells are normally passaged (i.e., most cells are latently infected), the virus is maintained as a latent episome, with highly restricted viral gene expression and lack of virus production. Chemically induced, viral gene expression switches from the latent program to an ordered cascade of lytic gene expression, leading to viral replication, virion production, cell lysis, and viral release (Renne et al., 1996a; Renne et al., 1996b; Sarid et al., 1998; Zhong et al., 1996). However, the classification of a viral gene as latent or lytic solely by analysis of RNA expressed in bulk PEL cultures has been complicated by the fact, that a characteristic small percentage of every cultured PEL population spontaneously undergoes lytic reactivation (Renne et al., 1996b; Zhong et al., 1996). To overcome this problem, *in situ* hybridization was performed with KS specimens, revealing that the *kaposin* gene (ORF K12, later referred to as *kaposin A* [Sadler et al., 1999]) was expressed in at least 85% of spindle cells, while ORF25/MCP, a lytic structural protein in PELs that is highly conserved in *Herpesviridae*, was expressed in no more than 10% of the spindle cells (Nealon et al., 2001; Staskus et al., 1997). Due to this approach, *kaposin* was classified as a latent gene, and provided a seminal paradigm for classifying expression of other KSHV genes (Staskus et al., 1997). Further genome-wide analyses of KSHV gene expression, utilizing PEL models, compared the gene transcription patterns of each viral ORF during normal culture of PELs to the response to TPA treatment and lytic viral induction (Sarid et al., 1998). On this basis each viral ORF was classified as class I (detected under standard growth conditions, no induction upon TPA treatment), class II (detected without TPA and further induced by TPA addition), or

class III (undetectable without TPA but induced by the chemical), respectively. This examination revealed a cluster of three class I genes, LANA-1 (latency-associated nuclear antigen-1), ORF72 (viral cyclin D) and K13 (fas-ligand IL-1 β-converting enzyme inhibitory protein [v-Flip]), whose wide expression in KS specimens confirms their latent classification (Davis et al., 1997; Dittmer et al., 1998). However, the detection of *kaposin A* as a class II gene in these cells demonstrates that not all latent genes are class I (Sarid et al., 1998; Sadler et al., 1999; Staskus et al., 1997). The group of class II genes typically consisted of herpesvirus regulatory and viral DNA replicative genes, as well as most of the viral homologs of cellular genes. The class III genes, in contrast, encoded primarily typical late (L) genes, such as viral structural and replication genes (Sarid et al., 1998). More recent studies based on DNA microarrays have enabled simultaneous comparisons of the transcription kinetics of quasi all KSHV genes (Dittmer, 2003; Jenner et al., 2001; Paulose-Murphy et al., 2001). Besides confirming the original PEL-based classifications of the viral genes based on the addition of TPA, microarrays are for example also a powerful means to determine the kinetics of first appearance and peak expression of the lytic genes.

Gene expression studies after reactivation of latent virus have identified immediate early (IE) transcripts (typical for regulatory genes of herpesviruses) based on their resistance to treatment with cycloheximide (Sun et al., 1999). One of these transcripts is the ORF50 (replication and transcriptional activator [Rta]), whose expression product is able to reactivate the virus from latency in PEL cells (Gradoville et al., 2000; Lukac et al., 1998; Lukac et al., 1999; Sun et al., 1998). The ORF50 is tricistronic and also encodes the downstream genes K8/K-bZIP/RAP and K8.1 (Gruffat et al., 1999; Lin et al., 1999; Lukac et al., 1998; Seaman et al., 1999; Sun et al., 1998; Sun et al., 1999; Zhu et al., 1999). Investigations of transcript architecture from individual loci revealed that numerous KSHV transcripts are spliced and many are polycistronic.

Interestingly, the low level of spontaneous lytic gene expression detected against the backdrop of latent expression in most PEL cultures is highly similar to what is found in KS clinical samples (Fakhari and Dittmer, 2002; Jenner et al., 2001; Paulose-Murphy et al., 2001; Sarid et al., 1998). This is most likely not an artefact of tissue culture models, since most infected cells in KS specimens display a latent KSHV gene expression with occasional cells expressing lytic transcripts (Chan et al., 1998;

Dupin et al., 1999; Katano et al., 2000; Lin et al., 1998; Orenstein et al., 1997; Parravicini et al., 2000; Staskus et al., 1997; Sun et al., 1999). More recent experiments of *de novo* infection of cultured endothelial cells have also demonstrated a similar mixture of latent and lytic gene expression (Ciufo et al., 2001; Lagunoff et al., 2002; Moses et al., 1999).

2.3.5 *Kaposin*

At the right end of the KSHV genome a cluster of latently expressed proteins can be found, where besides the latency-associated nuclear antigens, v-cyclin and v-FLIP also the K12 locus is located (Dittmer et al., 1998).
The K12 locus is divergent and consists of the K12 ORF and two upstream sets of 23 nucleotide direct repeats DR2 and DR1. Surprisingly, Sadler and colleagues presented evidence that these direct repeats are expressed on the protein level in KSHV-infected cells despite the absence of AUG start codons (Sadler et al., 1999). They immunized mice against PEL tumor cells to generate monoclonal antibodies and found that approximately half of the mabs were directed against DR repeats. By tagging the DR repeats at the 3' end, they could show that all reading frames are expressed and speculate that different *kaposin* protein isoforms are expressed initiating from distinct start codons using different reading frames. These isoforms derived either from ORF K12 itself or from the repetitive elements upstream of ORF K12 were termed *kaposin A*, *B*, and *C* (Fig. 6A). While *kaposin A* is initiated from the only predicted translational start codon within the locus, the AUG codon at the 5' end of K12, putative CUG or GUG alternative start codons, can be found in or 5' of the DR1 and DR2 repeats. Both direct repeat regions lack stop codons in all three reading frames. The open reading frames 2 and 3 run into stop codons between the DR repeats and ORF K12. In contrast, reading frame 1 is open to the 3' end of K12. Intriguingly, translation of DR2 and DR1 results in a 23-amino acid peptide of common sequence in all three reading frames (Fig. 6B). In Western blot analyses Sadler and colleagues detected proteins of 54, 48, 38 and 32 kd, of which *kaposin B* (containing the DR repeats but not K12) with a size of approximately 48 kd is the major protein expressed. Based on the structural sequence information and incited by these results, they hypothesized that (i) internal ribosomal entry is caused by the DR repeat region enabling the expression of K12, (ii) more translational products

may be produced, (iii) isoforms containing K12 sort to a different subcellular compartment as the other isoforms, (iiii) the different isoforms could produce differences in activity or stability, (v) one of the *kaposin* isoforms is a regulatory molecule whose expression at high levels is not compatible with cell survival or growth and that (vi) the complex translational control is mandatory to titrate expression levels of this toxic product down.

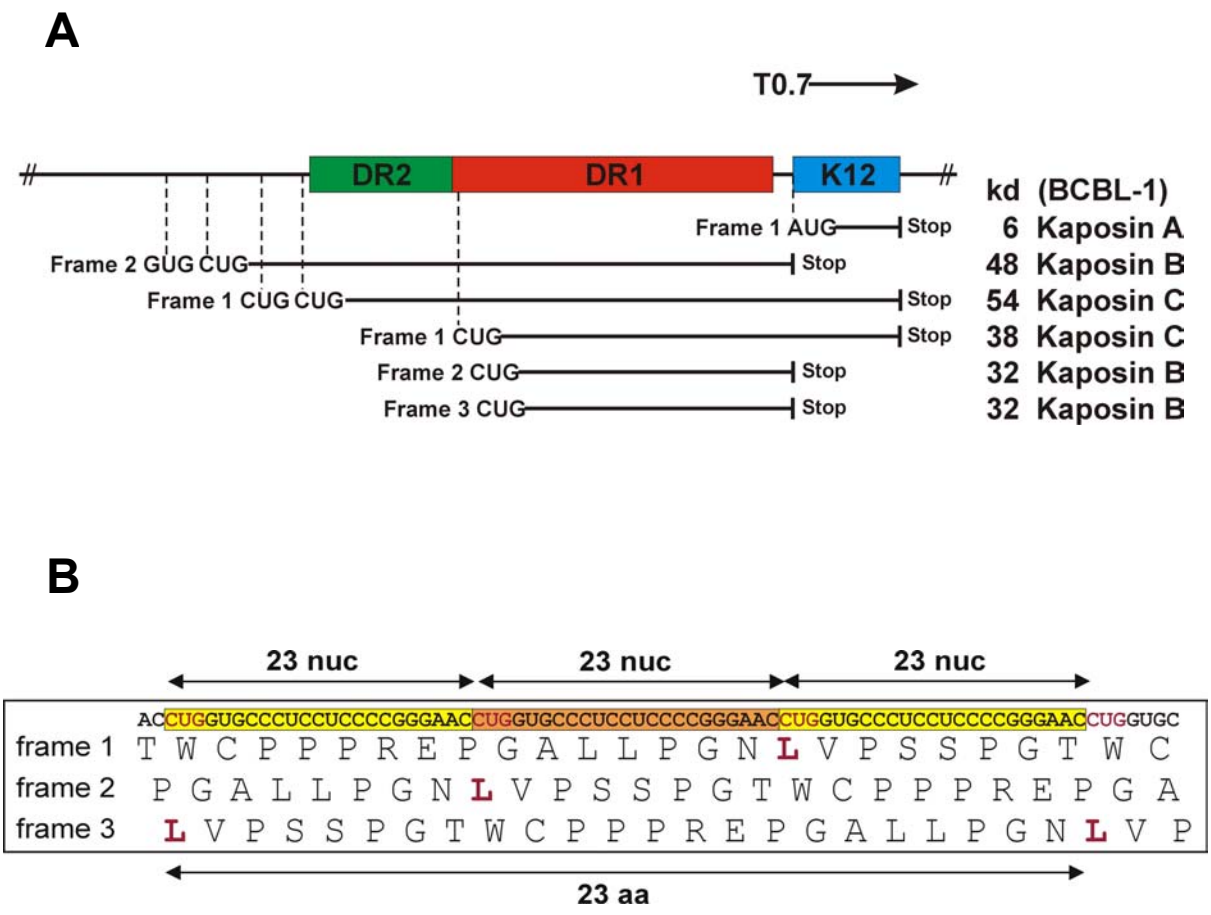


Fig. 6: Coding potential of the K12 locus

(A) CUG and GUG alternative translation initiation codons are indicated with the reading frame and size of the resultant translation products for a BCBL-1 mRNA. Additional CUGs are present within each DR1 repeat in all three reading frames. T0.7, see text (B) Translation of DR1 and DR2 (not shown) results in a repeating 23 aa peptide of common sequence in all three reading frames. The single letter code of DR1 is shown below the appropriate reading frame of the mRNA sequence. The 23 aa repeats are encoded by three 23-nt repeats (23 nuc). CUGs are shown in read. The leucine residue was randomly assigned as the start of each repeat and is coloured red in each reading frame (according to Sadler et al., 1999).

The genomic sequence between the start sites and the K12 ORF is highly polymorphic and varies markedly in number of direct repeats between different KS specimens and PEL cell lines. Transcription of this locus produces mRNAs that vary in length in different isolates (Sadler et al., 1999). Whereas the first identified gene product of this locus, *kaposin A*, was originally reported to be expressed by a 0.7 kb mRNA (T0.7), later reports identified several longer transcripts of up to 2.4 kb in KS and PEL cells harboring the upstream repeat regions. Therefore, the translation initiation of *kaposin A* at the AUG start codon of the K12 ORF was predicted to involve leaky ribosomal scanning or internal translational initiation from transcripts containing the upstream repetitive sequences (Sadler et al., 1999; Zhong et al., 1996). Recent data presented by Li and colleagues have identified a spliced transcript that includes a 5' non-coding exon derived from a region between ORFs 72 (v-cyclin) and 73 (LANA), approximately 5 kbp upstream of the 5' end of the previously identified *kaposin B/C* transcripts (Li et al., 2002). This splicing effect appears to be common to PEL and KS tissue and several PEL cell lines. It is thus possible that *kaposin* transcripts are produced from either of two promoters (Li et al., 2002; Sadler et al., 1999). Since the K12 locus expresses abundant *kaposin* transcript(s) during latency in KS tissue and PEL cells, but is also strongly induced following lytic reactivation, it was hypothesized that the encoded proteins may mediate functions that serve both replication modes (Sadler et al., 1999; Staskus et al., 1997; Sturzl et al., 1997; Zhong et al., 1996). The proximal *kaposin B/C* promoter driving the unspliced transcript is highly responsive to the immediate early ORF50 transactivator, which binds directly or indirectly to this region (Chang et al., 2002).

The finding that *kaposin* can be expressed during the latent phase of infection suggests that it contributes to KSHV-associated malignancies. This hypothesis was supported by the results from functional analyses of the hydrophobic 60 aa protein *kaposin A*, which was found to be transforming *in vitro* in Rat-3 fibroblasts and *in vivo* in nude mice (Kliche et al., 2001; Muralidhar et al., 1998). In transduced Rat-3 cells *kaposin A* was shown to be localized in the cytoplasm, and it was proposed that *kaposin A* is Golgi-associated (Muralidhar et al., 1998; Muralidhar et al., 2000). More recent data from confocal microscopy and subcellular fractionation experiments indicate that *kaposin A* has a predominantly perinuclear localization in PEL cells and transfected NIH3T3 cells. As indicated by *kaposin A*-specific immunostaining of non-permeabilised cells detected by flow cytometry, *kaposin A* can also distribute to the

plasma membrane (Kliche et al., 2001; Tomkowicz et al., 2002). This result coupled with secondary structure predictions and hydrophobicity plots for the 60 aa protein suggested that *kaposin A* is a type II transmembrane protein with an extracellular c-terminal domain (Kliche et al., 2001). The *kaposin A*-induced transformation is mediated through a direct interaction of *kaposin A* with cytohesin-1, a guanine nucleotide exchange factor (GEF) for ADP-ribosylation factors (ARF), which leads to an activation of MAP kinases. The transformed phenotype shown by actin remodeling, focus formation and gene activation, was reverted by a cytohesin-1 E157K mutant, which is deficient in catalyzing the guanine nucleotide exchange. *Kaposin A* was shown to activate cytohesin-1 by recruitment to the cell membrane, similar to phosphatidylinositol-mediated GEF recruitment and activation, which subsequently stimulates the ARF GTPase (Kliche et al., 2001).

2.4 Aim of this study

The K12 locus is a complex genomic region, which consists of the ORF K12 and two sets of upstream direct repeats. Whereas previous studies focused on *kaposin A* (ORF K12) and its function, little is known about the expression of other protein products originating from this locus (Kliche et al., 2001; Muralidhar et al., 1998). Sadler and colleagues showed that the upstream repeat region is expressed on the protein level in both, PEL cell lines and KS tumors (Sadler et al., 1999). They hypothesized that a variety of translational products is expressed from the K12 locus. Furthermore, they suggested that internal ribosomal entry is caused by the DR repeat region, that different isoforms may produce differences in activity or stability and that one or more of the *kaposin* isoforms are regulatory molecules whose expression is titrated by the complex translational control.

The aim of this work was to characterize biochemically and functionally the lytical *kaposin* protein isoforms generated in the PEL cell line BCBL-1. The concept of the present study was first to create molecular tools, qualifying to address the following questions: (i) the analysis and resolution of the expression pattern including the determination of the cellular localization, (ii) the biochemical characterization, (iii) the investigation of functional properties, (iv) the search for interaction partners and, finally, (v) the mutual influence of different isoforms on each other.

3 MATERIALS AND METHODS

3.1 Materials

3.1.1 Equipment

Bacterial Shaker	Kühner, Bürsfelden, Switzerland
Balances	Sartorius, Göttingen, Germany
Centrifuge GP	Beckman, Palo Alto, USA
Centrifuge J2-21	Beckman, Palo Alto, USA
Centrifuge Varifuge 3.0R	Heraeus, Hanau, Germany
Centrifuge Minifuge RF	Heraeus, Hanau, Germany
Centrifuge Labofuge T	Heraeus, Hanau, Germany
Centrifuge, refrigerated and non-refrigerated	Heraeus, Hanau, Germany
Confocal laser scanning microscope	Leica, Bensheim, Germany
Confocal laser scanner	Leica, Bensheim, Germany
Eagle eye	Stratagene, Amsterdam, The Netherlands
Elisa Reader	Tecan Labinstruments, Crailsheim, Germany
Film developing machine	Optimax Typ TR MS Laborgeräte, Heidelberg, Germany
Fluorescence/light microscope Axiovert 35	Zeiss, Oberkochen, Germany
Fluorescence/light microscope Axiovert 200M	Zeiss, Oberkochen, Germany
Fridge (4°C)	Liebherr, Ochsenhausen, Germany
Freezer (-20°C)	Liebherr, Ochsenhausen, Germany
Freezer (-80°C)	Forma Scientific, Inc., Marietta, Ohio, USA
Cryo 1°C Freezing Container	Nalgene Nunc, Wiesbaden, Germany
Gel dryer	Bio-Rad, Munich, Germany
GelAir drying system	Bio-Rad, Munich, Germany
Incubators for cell culture (37°C)	Forma Scientific, Inc., Marietta, Ohio, USA
Inverted microscope TMS	Nikon, Düsseldorf, Germany

Laminar Flow Hood Steril Gard II A/B3	The Baker Company, Sanford, Maine,USA
Magnetic stirrer with heating block	Janke & Kunkel, Staufen, Germany
Microwave	AEG, Berlin, Germany
Overhead mixer	Heidolph, Schwabach, Germany
PCR Thermal Cycler GeneAmp 2400	Perkin Elmer, Weiterstadt, Germany
pH-Meter	WTW, Weilheim, Germany
Photometer Gene Quant II	Pharmacia/LKB, Freiburg, Germany
Pipettes	Gilson, Villies Le Bel, France; Eppendorf, Hamburg, Germany
Pipetting aid	Technomara, Zürich, Switzerland
Electrophoresis Power supply EPS200	Amersham-Pharmacia, Freiburg, Germany
Sonifier 450	Branson Ultrasonics Corp., Danbury, USA
Thermomixer	Eppendorf, Hamburg, Germany
UV-transilluminator (366 nm)	Vetter, Wiesloch, Germany
(254 nm)	Konrad Benda, Wiesloch, Germany
Vortex mixer	IKA Works, Inc, Wirmington, USA
Water bath	Julabo, Seelbach, Germany GFL, Burgwedel, Germany

3.1.2 Chemicals

Acetic Acid	Roth, Karlsruhe, Germany
Acrylamide/Bisacrylamide 37,5/1 (Rotiphorese Gel 30)	Roth, Karlsruhe, Germany
Agar for plates	BD Biosciences Clontech, Heidelberg, Germany
Agarose electrophoresis grade	Invitrogen, Karlsruhe, Germany
Ammonium persulfate (APS)	Sigma, Munich, Germany
Ampicillin	Roche Diagnostics, Mannheim, Germany

Bacto peptone	BD Biosciences Clontech, Heidelberg, Germany
Bacto tryptone	BD Biosciences Clontech, Heidelberg, Germany
Bacto yeast extract	BD Biosciences Clontech, Heidelberg, Germany
Bicine	Sigma, Munich, Germany
Bromophenol blue	Serva, Heidelberg, Germany
Bovine serum albumin (BSA)	Sigma, Munich, Germany
Calcium chloride	Merck, Darmstadt, Germany
Chloramphenicole	Sigma, Munich, Germany
Coomassie brilliant blue R-250	Bio-Rad, Munich, Germany
Dextrose	BD Biosciences Clontech, Heidelberg, Germany
Dimethylsulfoxide (DMSO)	Merck, Darmstadt, Germany
Disodiumhydrogenphosphate	Merck, Darmstadt, Germany
Dithiothreitol (DTT)	Roth, Karlsruhe, Germany
dNTPs	Roche Diagnostics, Mannheim, Germany
DMF (N,N-dimethylformamide)	Sigma, Munich, Germany
DO (dropout) supplements	BD Biosciences Clontech, Heidelberg, Germany
Dulbecco's modified Eagle's medium (DMEM)	Gibco BRL, Karlsruhe, Germany
Ethanol (EtOH)	Riedel-de Haën, Seelze, Germany
Ethidium bromide	Sigma, Munich, Germany
Ethylenediamintetraacetate disodium salt (EDTA)	Roth, Karlsruhe, Germany
Ethylene glycol	Sigma, Munich, Germany
Fetal calf serum (FCS)	Gibco BRL, Karlsruhe, Germany
Glucose	Merck, Darmstadt, Germany
Glutathione-Sepharose 4B	Amersham-Pharmacia, Freiburg, Germany
Glycerol	Roth, Karlsruhe, Germany
Glycine	Serva, Heidelberg, Germany

Histogel	Linaris, Wertheim-Bettingen, Germany
Hydrochloric acid (HCl)	Merck, Darmstadt, Germany
Interferon (IFN) α	PBL Biomedical Laboratories, Piscataway, USA
Imidazole	Fluka, Seelze, Germany
Ionomycin	Sigma, Munich, Germany
Isopropanol	Riedel-de Haën, Seelze, Germany
Isopropylthio-b-D-galactosid (IPTG)	Roth, Karlsruhe, Germany
Kanamycin	Serva, Heidelberg, Germany
L-glutamine	Gibco BRL, Karlsruhe, Germany
L-Glutathione (reduced)	Sigma, Munich, Germany
Magnesium chloride	Merck, Darmstadt, Germany
Magnesium sulfate	Merck, Darmstadt, Germany
2-mercaptoethanol	Merck, Darmstadt, Germany
Methanol	Merck, Darmstadt, Germany
N-butyrate	Sigma, Munich, Germany
Nonidet P40 (NP-40)	Fluka, Seelze, Germany
Pefabloc	Roche Diagnostics, Mannheim, Germany
Polyethylene glycol (PEG 1000)	Sigma, Munich, Germany
Penicillin-Streptomycin	Gibco BRL, Karlsruhe, Germany
Phenylmethylsulfonfluoride (PMSF)	Roche Diagnostics, Mannheim, Germany
Phosphate buffered saline (PBS)	Dulbecco's Gibco BRL, Karlsruhe, Germany
Ponceau S	Sigma, Munich, Germany
Potassium acetate	Riedel-de Haën, Seelze, Germany
Potassium chloride	Merck, Darmstadt, Germany
Protein G-Sepharose Fast Flow	Amersham-Pharmacia, Freiburg, Germany
Rosswell Park Memorial Institute (RPMI)1640	Gibco BRL, Karlsruhe, Germany
SD Base medium	BD Biosciences Clontech, Heidelberg, Germany
Skim milk powder	Merck, Darmstadt, Germany

Sodium acetate	Riedel-de Haën, Seelze, Germany
Sodium azide	Serva, Heidelberg, Germany
Sodium chloride	Riedel-de Haën, Seelze, Germany
Sodium dodecylsulfate (SDS)	Merck, Darmstadt, Germany
Sodium hydroxid	J.T.Baker B.V., Deventer, Holland
Sorbitol	Sigma, Munich, Germany
Tetramethylethylenediamin (TEMED)	Amersham-Pharmacia, Freiburg, Germany
12-O-tetradecanoylphorbol-13-acetate (TPA)	Sigma, Munich, Germany
Tris(hydroxymethyl)aminomethan (Tris)	Roth, Karlsruhe, Germany
Triton X-100	Serva, Heidelberg, Germany
Trypsin	Gibco BRL, Karlsruhe, Germany
Tween 20	Merck, Darmstadt, Germany
Urea	Roth, Karlsruhe, Germany
Western Blue® Stabilized Substrate for Alkaline Phosphatase	Promega, Mannheim, Germany
X-α-Gal	BD Biosciences Clontech, Heidelberg, Germany

3.1.3 Additional materials

Autoradiography films BIOMAX-MR	Eastman-Kodak, Rochester, USA
Cell culture plastic ware	Greiner, Nürtingen, Germany Nunc, Wiesbaden, Germany Falcon/Becton Dickinson, Heidelberg, Germany
Filter paper (3 mm)	Whatman Ltd., Maidstone, England
Glass slides for IF	Marienfeld, Bad Mergentheim, Germany
Protran nitrocellulose transfer membranes	Schleicher & Schuell, Dassel, Germany
Sterile filter units	Millipore

3.1.4 Cell lines

293	human embryonal kidney cell line (ATCC: CRL-1573)
HeLa	human cervix carcinoma (ATCC :CCL-2)
BCBL-1	body cavity-based lymphoma cell line, kindly provided by Dr. Don Ganem, USCF, San Francisco, USA

3.1.5 Recombinant vaccinia viruses

Recombinant vaccinia virus expressing *kaposin A*, vKapA, was generated as reported previously (Kliche et al., 2001). Recombinant vaccinia virus vTF-7 expressing T7 polymerase was provided by the NIH AIDS reagent program (Fuerst et al., 1986).

3.1.6 Bacterial strains

DH5α	Gibco BRL, Karlsruhe, Germany
BL21 RIL	kindly provided by Dr. K.-P. Hopfner, Genzentrum, München, Germany

3.1.7 Yeast strains

AH109	BD Biosciences Clontech, Heidelberg, Germany

3.1.8 Plasmids

pBCBL-1-XhoII-NheI	kindly provided by Dr. Don Ganem, USCF, San Francisco, USA
p53wt	(Hoppe-Seyler and Butz, 1993)
pCDNA 3.1 zeo Grb2 f. l.	kindly provided by Dr. Hermann Schätzl, TU, München, Germany
pCR3	Invitrogen, Karlsruhe, Germany

pCRE-Luc	Stratagene, Amsterdam, The Netherlands
pCR3Ig0.2	(Kliche et al., 2001)
pCR3kapB	this study
pCR3kapD	this study
pCR3kapE	this study
pEGFP-C1	BD Biosciences Clontech, Heidelberg, Germany
pEGFP-kapB	this study
pEGFP-DR2	this study
pEGFP-DR1	this study
pEGFP-DR2-NLS	this study
pET-15b	Novagen, Madison, USA
pET-DR2	this study
pGADT7	BD Biosciences Clontech, Heidelberg, Germany
pGADT7-kapB	this study
pGADT7-DR2	this study
pGADT7-DR1	this study
pGADT7-Grb2-C-SH3	kindly provided by Dr. Hermann Schätzl, TU, München, Germany
pGBKT7	BD Biosciences Clontech, Heidelberg, Germany
pGBKT7-kapB	this study
pGBKT7-DR2	this study
pGBKT7-DR1	this study
pGBKT7-Grb2 f. l.	this study
pGBKT7-Grb2 C-SH3	this study
pGEX-4T-1	Amersham-Pharmacia, Freiburg, Germany
pGEX-DR2	this study
pGEX-DR1	this study
pHIVluc	(Holloway et al., 2000)
p-IL6	kindly provided by Gergana Iotzova, Genzentrum, München, Germany
pISRE-Luc	Stratagene, Amsterdam, The Netherlands
pNFκB-Luc	Stratagene, Amsterdam, The Netherlands
pUC21	New England Biolabs, Beverly, USA
pRK5c-mycRasV12	kindly provided by Dr.Alan Hall, MRC, London, UK

pRTU1 and pRTU14	kindly provided by Dr. Arndt Kieser, GSF, München, Germany
pSV_2tat72	NIH AIDS reagent program
pTIT-GFP	kindly provided by Dr. Karl-Klaus Conzelmann, Gene Center, München
p53-Luc	Stratagene, Amsterdam, The Netherlands
pSRE-Luc	Stratagene, Amsterdam, The Netherlands
pVEGF1-Luc	kindly provided by Dr. Werner Risau, MPI für physiologische und klinische Forschung, Bad Nauheim, Germany

3.1.9 Oligonucleotides

name	**sequence (5'→3')**
NsiI/3xStop/XhoI for	TGGATAGAGGCTTAACGTGAC
NsiI/3xStop/XhoI rev	TCGAGTCACGTTAAGCCTCTATCCATGCA
NLS NsiI/XhoI for	TCCCCAAGAAGAAGCGCAAGGTGTAGC
NLS NsiI/XhoI rev	TCGAGCTACACCTTGCGCTTCTTCTTGGGGATGCA

The oligonucleotides were obtained from metabion (Martinsried, Germany) and Thermo hybaid (Ulm, Germany).

3.1.10 Molecular weight markers

Gene Ruler 100 bp DNA ladder	MBI Fermentas, St. Leon-Rot, Germany
Gene Ruler DNA 1 kb ladder	MBI Fermentas, St. Leon-Rot, Germany
See blue plus 2 prestained protein standard low range	Invitrogen, Karlsruhe, Germany

3.1.11 Kits

BCA Protein Assay	Pierce, Rockford, USA
Dual-Luciferase® Reporter Assay System	Promega, Mannheim, Germany
ECL western blotting detection system	Amersham-Pharmacia, Freiburg, Germany
Effectene Transfection Reagent	Qiagen, Hilden, Germany
Luciferase Assay System	Promega, Mannheim, Germany
Pharmacia GFX PCR DNA Gel Purification Kit	Amersham-Pharmacia, Freiburg, Germany
Qiafilter Plasmid Maxi Kit	Qiagen, Hilden, Germany

3.1.12 Antibodies

3.1.12.1 Primary antibodies

kap-4F11(IgG_{2a})	rat mab against the c-terminal domain of K12 (Kliche et al., 2001)
kdr1-3C12(IgG_{2a})	rat mabs against DR1; this study
kdr1-8D10(IgG_1)	
kdr2-4C6(IgG_1)	rat mabs against DR2; this study
kdr2-6H8(IgG_1)	
3F10	rat mab against HA Tag, Roche Diagnostics, Mannheim, Germany
9E10	mouse mab against Myc Tag, Santa Cruz Biotechnology, Heidelberg, Germany
B-14	mouse mab against GST (B14), Santa Cruz Biotechnology, Heidelberg, Germany
C-16	rabbit polyclonal antiserum against 14-3-3γ, Santa Cruz Biotechnology, Heidelberg, Germany
M-20	goat polyclonal serum against lamin B , Santa Cruz Biotechnology, Heidelberg, Germany
SPA-860	rabbit polyclonal antiserum against calnexin, Stressgen Biotechnlogies Corp., BC, Canada

VAP-SV044	rabbit polyclonal antiserum against Grb2, Stressgen Biotechnlogies Corp., BC, Canada

3.1.12.2 Secondary antibodies

TIB173-FITC conjugated	mouse mab against rat IgG_{2a}
TIB170-biotinylated	mouse mab against rat IgG_1
alkaline phosphatase-conjugated:	
goat anti-rat	Jackson, Hamburg, Germany
peroxidase-conjugated:	
donkey anti-goat	Jackson, Hamburg, Germany
goat anti-rat	Jackson, Hamburg, Germany
goat anti-rabbit	Jackson, Hamburg, Germany
goat anti-mouse	Jackson, Hamburg, Germany

3.1.13 Enzymes

T4 DNA Polymerase	New England Biolabs, Beverly, USA
Calf Intestinal Alkaline Phosphatase (CIP)	New England Biolabs, Beverly, USA
T4 DNA Ligase	MBI Fermentas, St. Leon-Rot, Germany
AmpliTaq Gold® DNA Polymerase	Applied Biosystems, Foster City, CA, USA
T4 Polynukleotid kinase	New England Biolabs, Beverly, USA
Restriction Endonucleases	MBI Fermentas, St. Leon-Rot, Germany Roche Diagnostics, Mannheim, Germany New England Biolabs, Beverly, USA

3.2 Methods

3.2.1 Bacterial culture

3.2.1.1 Cultivation of bacteria

E. coli bacteria were grown in LB medium or on LB agar plates. Incubation was performed at 37°C with constant shaking.

LB medium (1 l):	10 g Bacto tryptone 5 g Bacto yeast extract 5 g NaCl
LB agar:	LB medium with 1.5 % agar
Selection medium:	LB medium with 100 µg/ml ampicillin and/or 50 µg/ml kanamycin

3.2.1.2 Preparation of competent bacteria

For preparation of competent bacteria a single clone of DH5α was picked and grown in 20 ml TYM medium at 37°C to an OD_{600nm} of 0.8. The bacterial culture was diluted with 100 ml TYM and incubated at 37°C until an OD_{600nm} between 0.5-0.9 was reached. Subsequently the culture was again diluted by adding 500 ml of TYM and incubated at 37°C. At an OD_{600nm} of 0.6 the culture was rapidly chilled down on ice water. The following incubations were all performed at 4°C or on ice. The bacteria were distributed to two 50 ml tubes and centrifuged 5 min at 3500 rpm (Heraeus Varifuge 3.0R). The supernatants were discarded and the pellets were resuspended in 100 ml icecold TfB I. After 40-50 min incubation on ice, the bacteria were centrifuged 10 min at 2500 rpm (Heraeus Varifuge 3.0R). The supernatants were discarded and the pellets were resuspended in 25 ml ice-cold TfB II. Aliquots of 0.4 ml were added to precilled 0.5 ml reaction tubes and stored at –80°C.

TYM:	10 mM $MgS0_4$ 100 mM NaCl 20 g/l Bacto tryptone 5 g/l Bacto yeast extract

TfB I:	30 mM KAc
	50 mM $MnCl_2$
	100 mM KCl
	10 mM $CaCl_2$
	15 % (v/v) Glycerol
TfB II:	10 mM MOPS pH 7.0
	75 mM $CaCl_2$
	10 mM $CaCl_2$
	15 % (v/v) Glycerol

both buffers sterilized by filtration (Ø 0.2 µm) and stored at 4°C.

3.2.1.3 Transformation

Different volumes of the ligation reaction mixture (5, 10, 20 µl) were added to 100 µl competent bacteria, mixed with 80 µl of 50 mM $CaCl_2$ and incubated 30 min on ice. After the heat shock, 1 min 42°C, 800 µl LB medium were added and bacteria were cultivated for 1 h at 37°C. Then 100 µl were taken and plated on LB agar plates with antibiotic(s). The residual bacteria were centrifuged (4000 g, 5 min), resuspended and plated the same way. The plates were incubated o/n at 37°C.

3.2.2 DNA techniques

3.2.2.1 Purification of plasmid DNA

Plasmid DNA was purified with the Pharmacia GFX Micro Plasmid Kit in small scale and the Qiafilter Plasmid Maxi Kit in large scale according to the manufacturer's instructions.

3.2.2.2 Determination of DNA concentration

The concentration and purity of the purified DNA was determined by measuring the UV absorbance at 260 and 280 nm. The DNA concentration was calculated with the OD_{260nm} (1 OD_{260nm} = 50 µg/ml dsDNA or 33 µg/ml ssDNA). The purity was estimated with the OD_{260nm}/OD_{280nm} ratio, with a ratio of approximately 1.8 indicating a low degree of protein contamination.

3.2.2.3 Restriction endonuclease digestion

Restriction endonuclease reactions were performed according to the manufacturer's recommendations. In general, 1.5 µg DNA were digested for 2 h at the respective temperature with 10-20 U enzyme. Efficacy of the cleavage reaction was controlled by agarose gel electrophoresis.

3.2.2.4 Oligonucleotide phosphorylation and annealing

Single stranded oligonucleotides were phosphorylated o/n at 37°C o/n with T4 Polynukleotid kinase.
Reaction mixture:
1.5 µl oligonucleotide (150 pMol)
2 µl 10 mM ATP
2 µl 10x PNK buffer (700 mM Tris-HCl (pH 7.6), 100 mM $MgCl_2$, 50 mM dithiothreitol)
1 µl T4 Polynukleotid kinase (10 U)
13.5 µl H_2O

For annealing the phosphorylation mixtures of complementary oligonucleotides were combined and diluted to 200 µl in H_2O. The reaction tube was boiled in 500 ml of H_2O for 5 min and allowed to cool down to RT. Subsequently, the oligos were precipitated by ethanol precipitation as described below and resolved in an appropriate amount of H_2O before used in ligation.

3.2.2.5 5'-Dephosphorylation reaction

5'-dephosphorylation reaction of plasmid vector DNA after restriction endonuclease cleavage was performed with the calf intestinal alkaline phosphatase (CIP). 50 U CIP were added to about 1.5 µg restriction enzyme digested plasmid DNA. After 30 min incubation at 37°C was stopped and the DNA was isolated by agarose gel electrophoresis.

3.2.2.6 Polymerase chain reaction (PCR)

Polymerase Chain Reaction (PCR) was performed with the AmpliTaq Gold® DNA polymerase from *Thermus aquaticus* to verify the cloning of the oligonucleotides (containing stop codons or a NLS, see 2.1.8) into the plasmids pCR3kapB and pEGFP-DR1-NLS, respectively.
The reaction mixture contained:
5 µl 10x PCR Buffer (100 mM Tris-HCl pH 8.3, 500 mM KCl, 15 mM $MgCl_2$, 0,01% gelatine w/v.)
1 µl 10 mM dNTPs (200 µM each)
1 µl forward primer (150 pMol)
1 µl reverse primer (150 pMol)
1 µl AmpliTaq Gold® (5U)
21 µl H_2O
+ 20 µl template DNA in H_2O (bacteria pools)
Bacteria colonies were picked with pipette tips from plates and transfered into a PCR tube containing 20 µl of H_2O. Subsequently, the tubes were boiled for 10 min at 94°C before adding the PCR reaction mixture.

The following cycles were performed:
1. 94°C 5 min
2. 94°C 1 min
3. 55°C 1 min
4. 72°C 2 min

} 10x with 1°C decrease per cycle to 45°C (touchdown), then 30x

5. 72°C min 10 min

3.2.2.7 Isolation of DNA fragments

DNA fragments were separated by agarose gel electrophoresis, stained with ethidium bromide and detected with UV light (366 nm). The gel slice containing the DNA fragments was cut out and the DNA was isolated using the Pharmacia GFX PCR DNA Gel Purification Kit according to the manufacturer's instructions.

3.2.2.8 Phenol/chloroform extraction and ethanol precipitation

Proteins were removed from DNA preparations by extracting twice with 1x volume phenol/chloroform and once with 1x volume chloroform. After vigorous vortexing for 10 s the solution was centrifuged at 14000 rpm (microcentrifuge) for 1 min and the upper DNA containing phase was recovered. Then 0.1x volume 3 M NaAc pH 5.2 and 2.5x volume 100% EtOH (cold) were added, and incubation at –80°C was performed for 20 min. The precipitated DNA was centrifuged down at 14000 rpm for 30 min (4°C). Then the pellet was washed once with 70% EtOH (cold). After another centrifugation step (14000 rpm, 15 min, 4°C, microcentrifuge) the EtOH was carefully removed, the pellet air-dried at RT and finally resuspended in H_2O.

3.2.2.9 Ligation

For ligation about 50 ng vector DNA was used with a molar ration of vector/insert of about 1:3. The reaction was performed in a total volume of 20 µl 1x reaction buffer (MBIFermentas) with 5 U T4 DNA Ligase (MBI Fermentas). First vector and insert were mixed in reaction buffer, then the ligase was added. After incubation o/n in a watherbath at 16°C the ligation either directly transformed into competent bacteria or stored at –20°C until further usage.

3.2.2.10 Agarose gel electrophoresis

Analysis of DNA fragments and plasmids was performed by agarose gel electrophoresis in 1x TAE. In general, agarose concentration was between 1 and 3 % in 1x TAE. The agarose was solubilized by heating in a microwave oven. Ethidium

bromide was added to a final concentration of 0.25 µg/ml (2,5 µl stock to 100 ml) just before pouring the gel. Probes were mixed with 0.17x volume loading buffer. Gels (6.5 x 9.5 cm) were run horizontally at 80-120 V. DNA was detected with UV light, λ=254 nm or λ=366 nm to cut out specific fragments.

loading buffer (6x in water)	MBI Fermentas, St. Leon-Rot, Germany
20x TAE:	800 mM Tris
	400 mM NaAc
	40 mM EDTA
	adjusted to pH 7.8 with acetic acid
Ethidium bromide (stock):	10 mg/ml

3.2.2.11 Plasmid construction

(1) *pCR3kapB*. A fragment containing the DR2 and DR1 repeat regions was subcloned from pBCBL-1-XhoII-NheI into pUC21 (New England Biolabs) using HindIII and NsiI restriction sites. Stop codons in each reading frame were added by subcloning the two oligos TGGATAGAGGCTTAACGTGAC and TCGAGTCACGTTAAGCCTCTATCCATGCA as adapters into the NsiI and XhoI restriction sites of this plasmid. Subsequently, a fragment excised by HindIII and XhoI was subcloned into pCR3 (Invitrogen). (2) *pCR3kapD*. The DR1 repeats were excised from pEGFP-DR1 by PstI and XhoI restriction sites and subcloned into a pCR3 derivative containing a HA Tag, in which K12 fragment excised with NsiI and XhoI from pBCBL-1-XhoII-NheI has been subcloned. (3) *pCR3kapE*. The DR1 repeats (containing an AUG start codon and a HA Tag) were subcloned by BglII and XhoI restriction sites from pGADT7-DR1 into pCR3. (4) *pEGFP-kapB*. The fragment excised by PstI and XhoI from pCR3kapB was subcloned into pEGFP-C1 (Clontech). (5) *pEGFP-DR2*. pEGFP-kapB was digested with SmaI and religated. (6) *pEGFP-DR1*. pEGFP-kapB was digested with HhaI, blunted with T4 DNA Polymerase and digested with XbaI. Subsequently, the fragment was ligated into pEGFP-C1 digested with SmaI and XbaI. (7) *pEGFP-DR1-NLS*. The DR1 repeats were excised by digestion of pEGFP-DR1 with PstI and XhoI and subcloned into pUC21. Subsequently, the oligos TCCCCAAGAAGAAGCGCAAGGTGTAGC and

TCGAGCTACACCTTGCGCTTCTTCTTGGGGATGCA encoding a SV-40 NLS and a stop codon were subcloned as adapters into the NsiI and XhoI restriction sites. The DR2-NLS fragment was eventually subloned by PstI and SacII into pEGFP-C2. (8) *pGADT7-kapB*. The fragment excised by EcoRI and XhoI from pEGFP-kapB was subcloned by EcoRI and XhoI restriction sites into pGADT7 (Clontech). (9) *pGADT7-DR2*. The EcoRI and XhoI fragment from pEGFP-DR2 was subcloned by EcoRI and XhoI restriction sites into pGADT7. (10) *pGADT7-DR1*. The fragment excised by EcoRI and XhoI from pEGFP-DR1 was ligated into EcoRI/XhoI digested pGADT7. (11) *pGBKT7-kapB*. (12) *pGBKT7-DR2*. (13) *pGBKT7-DR1*. Fragments isolated from pEGFP-kapB, pEGFP-DR2 and pEGFP-DR1 by EcoRI and XhoI digestion were subcloned into pGBKT7 (Clontech) digested with EcoRI and SalI. (14) *pGBKT7-Grb2 f. l.*. Grb2 f.l. was excised by BamHI and XhoI digestion from pCDNA 3.1 zeo Grb2 f. l. and ligated into BamHI/SalI digested pGBKT7. (15) *pGBKT7-Grb2-C-SH3.* Likewise, Grb2-C-SH3 was excised by BamHI and XhoI digestion from pGADT7-Grb2-C-SH3 and ligated into BamHI/SalI digested pGBKT7. (16) *pGEX-DR2*. (17) *pGEX-DR1*. Repeat regions isolated from pEGFP-DR2 and pEGFP-DR1 by EcoRI and XhoI digestion were subcloned into pGEX-4T-1 (Amersham). (18) *pET-DR2*. DR2 repeats were excised by PstI/XhoI digestion of pEGFP-DR1 and ligated into pUC21 via the same restriction sites. From this construct the DR2 repeats were subcloned by NdeI and XhoI restriction sites into pET-15b (Novagen).

3.2.3 Tissue culture

3.2.3.1 Cultivation and cryoconservation

The KSHV-infected PEL cell line BCBL-1 was cultured in RPMI 1640 supplemented with 20% fetal calf serum, 100 IU/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine. For induction of the lytic viral cycle BCBL-1 cells were treated for 48 h with 3 mM n-butyrate. 293 and Hela cells were cultured in DMEM/10% FCS plus supplements at 37°C and 5% CO2. For cryoconservation cells were detached with trypsin and centrifuged at 300 g for 5 min at 4°C. Then the cells were resuspended in 1 ml FCS/10% DMSO (4°C) with a final concentration of 0.5-1x10^7 cells/ml and transferred to cryovials which were cooled to –80°C in a “Cryo 1°C Freezing Container”. From there the vials were transferred to liquid nitrogen for longterm

storage. Frozen aliquots were quickly thawed at 37°C in a waterbath, 10ml DMEM was added and after centrifugation at 300 g for 5 min the supernatant was removed. Subsequently cells were resuspended in complete medium and transferred to cell culture dishes.

3.2.3.2 Calcium phosphate transfection

For transient transfection cells were grown on 10 cm Ø dishes to 60-70% confluency. 500 µl of 2x HBS pH 7.05 was added to a 15 ml Falcon tube. In another tube 20 µg DNA was combined with 500 µl 250 mM $CaCl_2$. The tube with the 2x HBS was vortexed while the DNA/$CaCl_2$ solution was added dropwise. The solution was incubated at RT for 15-20 min to allow the formation of the Calcium-DNA precipitate. Subsequently, the suspension was mixed with 6 ml fresh medium and was added to the cells after removal of the old medium. The next day protein expression was assessed by immunofluorescence.

2x HBS pH 7.05:	50 mM HEPES
	1.5 mM Na2HPO4x 2 H2O
	280 mM NaCl
	12 mM Glucose

3.2.3.3 Immunofluorescence

BCBL-1 cells that have been induced for 48 h with 3 mM n-butyrate were spotted onto poly-L-lysine-coated coverslips. Hela cells were grown on coverslips. Cells were fixed with ice-cold methanol for 2 min and subsequently blocked against non-specific binding for 1 h with PBS/2,5% FCS. The cells were incubated with the primary antibody diluted in PBS/2,5% FCS for 1 h, washed four times with PBS and incubated with the secondary antibody (fluorescein conjugated or biotinylated mouse anti-rat) for 1 h, followed by another washing step and subsequent incubation with Streptavidin Texas Red and/or Hoechst dye (to counterstain nuclear DNA). After a final washing step, the coverslips were mounted on glass slides with Histogel. The mounted cells were analysed using an inverse fluorescence/light microscope.

3.2.3.4 Reporter gene analysis

For luciferase reporter assays, 293 cells were split into 12-well plates the day before transfection. All plasmids were transiently transfected using the Effectene Transfection Reagent (Qiagen) according to the manufacterer's instructions. In all experiments, total amounts of transfected DNA were equalized between wells using empty pCR3. One day post transfection, cells were starved in DMEM, containing 1% fetal calf serum (plus TPA+Ionomycin [500 ng/ml+1 µM], IFNα [500 U/ml] or forskolin [30 µM] if used as positive control), for another 24 h. After 48 h cells were harvested by flushing them off the the plates with 700 µl of cold PBS, transfered to a tube and centrifuged at 2000 rpm for 2 min (4°C, cooled microcentrifuge). Supernatants were discarded and luciferase activity was determined by using a commercial luciferase assay system (Promega) and a tube luminometer (Berthold) according to the manufacterer's instructions.

3.2.4 Protein techniques

3.2.4.1 Cellular fractionation

For particular fractionation, stimulated BCBL-1 cells and transfected 293 cells were washed twice with PBS and resuspended in hypotonic lysisbuffer (10 mM Hepes, 10 mM KCl, 10 mM $MgCl_2$, 0.5 mM EDTA with 1 mM PMSF and 2 mM Pefablock) (according Nagel et al., 1998). After an incubation of 10 min on ice, cells were sheared by passing through a 26 gauge needle. To separate nuclei, lysates were centrifuged 10 min at 4°C and 110 g, the pellets were washed three times with F-actin buffer (10 mM HEPES pH 7.5, 0.5 mM ATP, 0.5 mM dithiothreitol, 20µM $MgCl_2$, 15% Glycerol) and resuspended in TE buffer (10 mM Tris, 1 mM EDTA pH 8). The postnuclear supernatant was ultracentifuged for 1 h at 4°C and 40,000 rpm and cytosolic supernatants were collected. The pellets were resuspended and washed with hypotonic lysis buffer and centrifuged for 10 min at 4°C and 14000 rpm. The resulting pellet was resuspended in hypotonic lysisbuffer containing 1% (vol/vol) Nonidet P-40, incubated on ice for 10 min and centrifuged again. Supernatants representing the detergent-soluble membrane fraction were collected, pellets representing the unsoluble fraction were resuspended in 2xSDS protein sample

buffer (10 % Glycerol, 0.2 % bromophenol blue, 4 % SDS, 4 % 2-mercaptoethanol, 50 mM Tris pH 6.8). Fractions were directly analysed by SDS-PAGE or stored at -20°C.

3.2.4.2 Co-immunoprecipitation

Co-immunoprecipitation was performed using the plasmids pGBKT7 and pGADT7 with T7 promoter and recombinant vaccinia virus vT7 expressing the T7 RNA polymerase. 293 cells were cultured on 10 cm dishes and infected with vTF-7 at a MOI of 10 in serum-free medium. 1,5 h after infection, cells were transfected with 10 µg of each of the two expression plasmids by calcium phosphate transfection. Expression was controlled using a GFP plasmid under the control of a T7 promoter (pTIT-GFP). After 24 h, cells were lysed by incubation in 1 ml of NP-40 lysis-buffer (1% NP-40, 140 mM NaCl, 5 mM $MgCl_2$, 20 mM Tris pH 7,6, 1 mM PMSF) for 30 min on ice. Lysates were centrifuged for 10 min at 20,500 g and 4°C to remove unsolubilized material and precleared with 50 µl of preequilibrated protein G-Sepharose. Subsequently, proteins were precipitated from the supernatant by adding 200 µl hybridoma supernatant of the specific anti-DR monoclonal antibodies (mab) and 50 µl of protein G-Sepharose beads and incubating in an overhead mixer o/n at 4°C. Beads were washed three times with ice-cold NP-40 buffer. Co-immunoprecipitations, cellular subfractions and total cell lysates of BCBL-1 and transfected 293 cells were resuspended in 2xSDS protein sample buffer. Cellular subfractions and total cell lysates were additionally sonicated for 30 s. Samples were boiled for 5 min and directly analysed by SDS-PAGE or stored at –20°C. For the equilibration of protein G-Sepharose, 1.5 g were washed 3x and resuspended with NP-40 lysis-buffer to obtain a 50% slurry.

3.2.4.3 Pull-down of recombinant SH3 domain proteins

For pull-down experiments of recombinant GST-SH3 domain proteins (kindly provided by Dr. Stephan Feller, Oxford, UK) approximately 600 ml of n-butyrate induced BCBL-1 cells were lysed in 10 ml of NP-40 buffer. Subsequently the lysate was divided into 20x 500 µl aliquots and each aliquot was incubated in an overhead

mixer with 45 µl of protein G-Sepharose, 50 µl of kdr1-8D10 hybridoma supernatant and 10 µg recombinant protein each at 4°C o/n. Beads were washed three times with ice-cold NP-40 buffer and resuspended in 2xSDS protein sample buffer. After boiling for 5 min, samples were analysed by SDS-PAGE on 15% gels. Precipitated proteins were detected by Western blotting with an primary anti-GST antibody and a peroxidase-conjugated secondary antibody (see below).

3.2.4.4 SDS PAGE

Gel electrophoresis was performed with minigels using the Protean II system (Bio-Rad) with 12 to 20% gels (80 x 50 x 1 mm). The solution for generating the separation gel was mixed and after pouring, the gel was overlaid with isopropanol. After polymerization the isopropanol was sucked of the gel. The stacking gel solution was poured on top of the separation gel and a comb was fixed. After polymerization the glass plates containing the gel were assembled in the gel electrophoresis apparatus. Samples or pellets from immunoprecipitation were resuspended in the appropriate amount of 2xSDS protein sample buffer and heated for 5 min to 95°C. After cooling to RT the samples were centrifuged for 2 min at 14000 rpm (microcentrifuge) and loaded on the gel together with a protein standard. Separation was performed at 150 V constant current for 1-2 h.

Separation Gel :	12%	15%	20%
Acrylamide/ Bisacrylamide (37.5:1)	2 ml	2.5 ml	3.33 ml
1.5 M Tris pH 8.8	1.25 ml	1.25 ml	1.25 ml
10 % SDS	50 µl	50 µl	50 µl
H_2O	1.675 ml	1.175 ml	0.343 ml
10 % APS	20 µl	25 µl	25 µl
TEMED	2.5 µl	2.5 µl	2.5 µl

Stacking gel:	5%
Acrylamide/ Bisacrylamide (37.5:1)	1.35 ml
0.5 M Tris pH 6.8	0.625 ml
10 % SDS	25 µl
H_2O	1.53 ml
10 % APS	12.5 µl
TEMED	2.5 µl

Electrophoresis buffer (10 x):	50 mM Tris
	384 mM glycine
	0.1% SDS

3.2.4.5 Western blotting

Proteins were blotted on nitrocellulose membranes using the Protean II system (Bio-Rad). A piece of nitrocellulose membrane and two pieces of filter paper and two sponges of the same size as the gel were soaked with transfer buffer. A sponge, a piece of filter paper, the nitrocellulose membrane, the gel, another piece of filter paper and again a sponge were packed. Subsequently, air bubbles were removed by rolling a test tube over the sponge and the package was clamped into the transfer tank with the nitrocellulose facing the anode. Blotting was performed with 100 V constant for 1 h. Proteins were detected after 2 min incubation in Ponceau staining solution. The membranes were labeled with a pen and was washed several times with H_2O to remove the Ponceau staining solution. Unspecific binding sites were blocked by incubation in TBST (TBS, 0.05% Tween 20), 5% skim milk powder, 0.02% NaN3 either 1 h at RT or o/n at 4°C. Then incubation with the first antibody was performed in 5-10 ml TBST (used also in the following washing and incubation steps) at 4°C o/n. After five washing steps of 15 min with approximately 200 ml buffer each, incubation with the secondary antibody coupled to peroxidase was performed in 15 ml buffer at RT for 1 h followed by washing 5x 10 min in 200 ml buffer.The blotted proteins were detected using the ECL Western blotting detection system (Amersham-Pharmacia) according to the manufacturer's instructions. The membrane was exposed to BIOMAX-MR autoradiography films for different time periods and films wre developed using an automatic film developing machine.

Transfer buffer (1l):
Tris base 5.8 g
Glycine 2.9 g
SDS 0.37 g
Methanol 200 ml
H_2O to 1l

Ponceau solution (100 ml):
Ponceau S 0.5 g
Glacial acetic acid 1 ml
H_2O 98.5 ml

3.2.4.6 Purification of recombinant DR2 and DR1 GST-tagged fusion proteins

To generate monoclonal antibodies, DR1 and DR2 repeats were expressed as GST-fusion proteins. Therefore 100 ml of o/n cultures of BL 21 containing the pGEX-DR2 and the pGEX-DR1 construct, respectively, were diluted with 500 ml prewarmed selection medium (LB with 100 µg/ml ampicillin) to an OD_{600nm} of 0.1 and grown at 37°C to an OD_{600nm} of 0.5. Subsequently, 500 ml of the cultures were induced with an IPTG concentration of 1 mM, followed by a 3 h incubation at 37°C. After incubation the cultured bacteria were pelleted by centrifugation at 4000 g for 20 min and resuspended in 30 ml PBS containing 1 mM PMSF and 2 mM Pefablock at 4°C, before being sonicated 3x for 20 s (output control level 7, 100 %) with a Branson Sonifier 450. 1 ml of 10 % Triton X-100 was added, solutions were shaken for 30 min at 4°C and centrifuged for 10 min at 4000 g following passage of the supernatants through a 0.45 µm filter. Cell extracts were combined with 2 ml of 50 % slurry of the Glutathione-agarose resin in PBS for each 100 ml of bacterial culture used to make the protein extract and the mixture was shaken for 1 h at 4°C. Beads were centrifuged down at 500 g for 5 min at 4°C and washed 3x with 10 bed volumes of PBS containing 1 % Triton X-100. GST-fusion proteins were eluted from the washed beads by adding one bed volume of Glutathione elution buffer (10 mM reduced Glutathione, 50 mM Tris-HCl pH 8.0) to the pellet. After incubation with gentle agitation for 10 min at RT beads were centrifuged again and the supernatants (which contain the eluted fusion proteins) were transfered to a fresh tube (this was repeated 2 times). Eluted fractions were analysed by SDS-PAGE on 12 % gels.

3.2.4.7 Purification of recombinant His-tagged DR2 fusion protein

20 ml of an o/n culture of BL21 RIL containing the pET 15b DR2 construct were added to 3 l of prewarmed selection medium (LB with 100 µg/ml ampicillin, chloramphenicole 100 µg/ml) and grown at 37°C to an OD_{600nm} of 0.4-0.5. 3ml 1 M IPTG was added to the medium followed by a 4 h incubation at 30°h. Bacteria were pelleted by centrifugation at 4000 g for 20 min and resuspended in 3 ml/g buffer 1 (200 mM NaCl, 3 mM imidazole, 20 mM Tris , pH 7. 6 including 1 mM PMSF) at 4°C. Subsequently the resuspension was sonicated 3x for 2 min (output control level 6) on ice water and centrifuged at 10000 g for 30 min and 4°C. The supernatant was used as mentioned below and a sample of the pellet was resuspended in 10 M urea and stored for SDS-PAGE analysis. Approximately 2-3 ml Ni-agarose beads were washed in 10 ml buffer 5 (200 mM NaCl, 300 mM imidazole, 20 mM Tris , pH 7. 6 including 1 mM PMSF), the suspension was loaded to a column and the beads were equilibrated with 6 bed volumes of buffer 1. The supernatant of the sonicated bacterial solution containing the soluble fusion protein was loaded on the column and washed with 4 bed volumes of buffer 1. Hereafter the HisTag-fusion protein was eluted with each 2x 5 ml buffer 2, 3, 4 (composition similar as buffer 1 but with 50 mM, 100 mM, 150 mM imidazole, respectively) and 5. Eluted fractions and samples of solution steps were analysed by SDS-PAGE on 12% gels.

3.2.4.8 Coomassie blue staining

For Coomassie blue staining of proteins, SDS-PAGE gels were incubated in Coomassie blue staining solution for 1-12 h and destained with 30% methanol/10% acetic acid by changing the destaining solution until the desired protein staining was visible.

Coomassie blue staining solution:	0.25% Coomassie brilliant blue R-250
	45% methanol
	10% acetic acid

3.2.4.9 Generation of rat monoclonal antibodies

Rat monoclonal antibodies were generated by Elisabeth Kremmer, GSF, Munich, Germany (Kremmer et al., 1995). Lou/C rats were immunized 3x with 50 µg DR1-GST-fusion protein at a time in intervals of three weeks. The first injection was done with complete Freund's adjuvant, the second with incomplete Freund's adjuvant both intraperitoneally and subcutaneously and the third without adjuvant intraperitoneally. For DR2 the procedure was similarly as described above, performing the first immunization with DR2-GST-fusion protein. However, for the second and third injection DR2-HisTag-fusion protein was used. Fusion of rat immune spleen cells with the myeloma cell line P3X63Ag8.653 was performed following the protocol of Köhler and Milstein 3 days after the final boost (Kohler and Milstein, 1992). Supernatants from hybridoma cells were tested by Western blotting for the presence of anti-DR2 and anti-DR1 antibodies, respectively. SDS-PAGE gels with lysates of induced BCBL-1 cells were blotted onto nitrocellulose, and unspecific binding sites were blocked by incubation in TBST (TBS, 0.05% Tween 20), 5% skim milk powder, 0.02% NaN3 for 1 h at RT. Thereafter the membranes were cut into small strips and each strip was put into a separate slot of a multi-slot chamber (selfconstructed). The Hybridoma supernatants were added and incubated o/n at 4°C with constant shaking followed by 3x 15 min washing steps with TBST and incubation with an alkaline phosphatase-conjugated goat anti-rat antibody (1:1000 in TBST diluted). Finally, bound antibodies were detected using the Western Blue® Stabilized Substrate for Alkaline Phosphatase (a mixture of BCIP and NBT in a proprietary stabilizing buffer, Promega) according to the manufacterer's instructions. Hybridoma cells producing antibodies recognizing DR2 or DR1 repeats were subcloned at least twice by limiting dilution. The immunoglobulin-isotypes were determined by ELISA.

3.2.5 Yeast culture

3.2.5.1 Competent yeast cells

To produce competent yeast cells, a preculture was prepared first. 10 ml YPD-medium were inoculated with one colony of the yeast strain AH109 overnight at 30°C. The following day, the preculture was added to 250 ml of fresh YPD-medium

and grown at 30°C until it reached an OD_{600} of 0.6. Cells were harvested in 50 ml Falcon tubes by centrifugation at 930 g for 5 min at 4°C. The supernatant was discarded and the cells were resuspended in 12.5 ml SBEG-solution each and subsequently pelleted another time. The resulting cell pellet was resuspended in 500 µl SBEG-Solution and parted into 100 µl aliquots. The aliquots were shock-frozen in liquid nitrogen and stored at – 80°C.

YPD medium (1 l):

For liquid medium:	10 g Bacto yeast extract
	20 g Bacto peptone
	20 g Dextrose
	add H_2O to 1 liter, autoclave
For solid medium:	add 20 g agar before autoclaving

SBEG-solution:	1 M Sorbitol
	10 mM Bicine pH 8,35 sterile
	3 % Ethylene glycol

3.2.5.2 Transformation and test of protein interaction

For transformation, a microlitertube with 100 µl competent AH109 yeast cells was thawed quickly in a waterbath at 37°C. Subsequently 1 µg of the used bait- (pGBKT7) and prey- (pGADT7) constructs was pipetted into the thawed yeast cells and mixed carefully with a pipet. 750 µl PEG/Bicine-solution were added, the suspension was mixed again and the reaction was incubated at 30°C for 1 h, followed by incubation for 5 min at 45°C. The next step was to pellet the cells for 2 min at 2700 g in a table centrifuge. The supernatant was removed by a pasteurpipet and the pellet was resuspended in 1ml NB-buffer. Again cells were pelleted, as described above, but only 800 µl of the supernatant were removed.

PEG/Bicine-Lösung:	40 % PEG 1000
	200 mM Bicine, pH 8,35, sterile

NB-Puffer: 0,15 M NaCl
10 mM Bicine pH 8,35, sterile

The pellet was resuspended in the remaining 200 µl supernatant and plated on SD/-Leu/-Trp plates, consisting of SD Base medium (+ 2% Agar) containing different formulation of dropout (DO) supplement (amino acid mixture, lacking the indicated amino acids). In pGBKT7, which has a Trp1 nutricion marker, the bait gene is expressed as a fusion to the GAL4 DNA-binding domain, while the prey gene is expressed in pGADT7 (Leu2 nutricion marker) as a fusion to the GAL4 activation domain. For detection of protein-protein interactions of the expressed constructs, colonies were replated on SD/-Ade/-His/-Leu/-Trp/X-α-gal, and positive clones were detected by growth and by α-galactosidase activity according to the Matchmaker Gal4 Two-Hybrid System 3 User Manual (Clontech, 1999).

4 Results

4.1 Expression of the DR2 and DR1 repeat region as GST-fusion proteins in *E. coli*

For examinations concerning the biochemistry of proteins, monoclonal antibodies are indispensable molecular detection tools used in a vast number of techniques. To start the investigations on the *kaposin* protein isoforms, the first step was to rise mabs against the repeat regions of the K12 locus, since only mabs against the c-terminus of the ORF K12 (*kaposin A*) were present at the beginning of this study.

For this purpose the DR2 and the DR1 repeat regions were expressed in *E.coli* as Glutathione-S-Transferase (GST)-fusion proteins. After induction with IPTG, a significant part of the bacterial proteins was contributed by the DR-GST-fusion protein (shown for DR1, Fig. 7A). Both of the recombinant proteins were found to be soluble in sufficient amounts and were purified by affinity chromatography using Glutathione-agarose resin (Fig. 7B). Although the purification was not complete, the proteins were sufficiently pure for immunization of Lou/C rats.

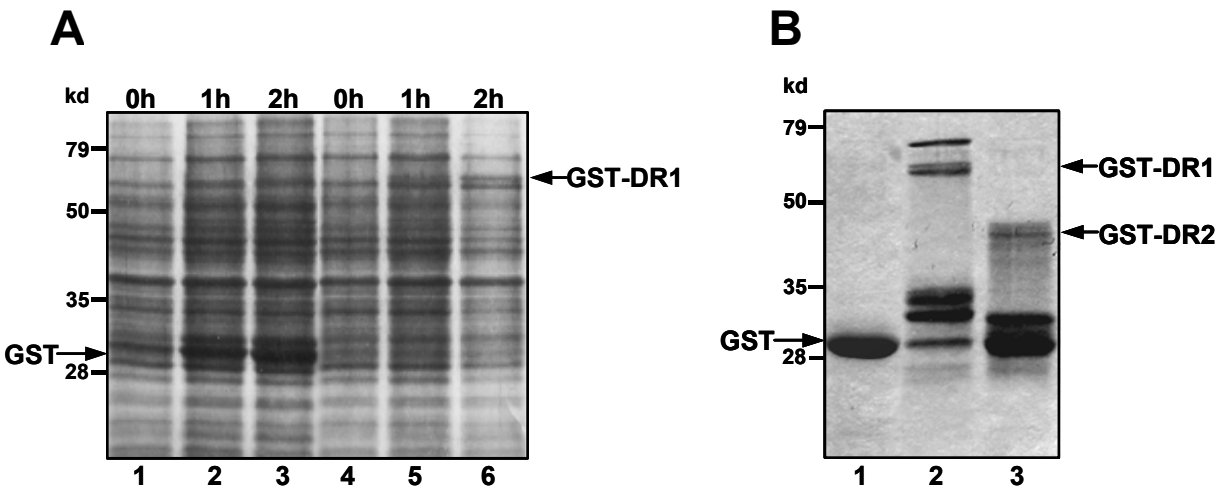


Fig. 7: Expression and purification of DR-GST-fusion proteins

(A) Expression of GST-DR1 in *E. coli*. The DNA encoding DR1 repeats was cloned into the bacterial expression vector pGEX-4T-1 and the GST-fusion protein was expressed as described in Materials and Methods (the same procedure was carried out for DR2 repeats, data not shown). Supernatants of *E. coli* cell lysates (after sonication) from 0-2h IPTG induced cultures were analysed by SDS-PAGE (lanes 1-3, pGEX-4T-1 transformed control culture; lanes 4-6, pGEX-DR1 transformed culture). Specific bands are indicated by arrows. (B) Elution of recombinant DR1- and DR2-GST-fusion proteins. Recombinant GST-fusion proteins were eluted from the Glutathione-agarose resin as described in Material and Methods and analysed by SDS-PAGE (lane 1, GST; lane 2, GST-DR1; lane 3, GST-DR2). Specific bands are indicated by arrows.

4.2 Expression of the DR2 repeat region as a HisTag-fusion protein in *E. coli*

An earlier attempt to boost the immunized rats with the recombinant GST-DR2-fusion protein was not successful. To eliminate the clones directed against the Tag sequence and also to enhance the specific immune response against DR2, the boost of the GST-DR2 immunized rats in this approach was performed with a DR2-HisTag-fusion protein. Thus the DR2 repeat region was expressed as a recombinant Histidine-tagged protein in *E. coli*. The transformed bacteria expressed the approximately 20 kd DR2-fusion-protein after IPTG induction. After lysis of the bacteria, it was found in the supernatant in reasonable amounts. Subsequently, the recombinant DR2-His-Tag-fusion protein was purified from the lysis supernatant via affinity chromatography using a Ni-agarose beads column (Fig. 8). An adequate purity level was reached, and the recombinant protein of fraction 8 (lane 12) was used for the boost.

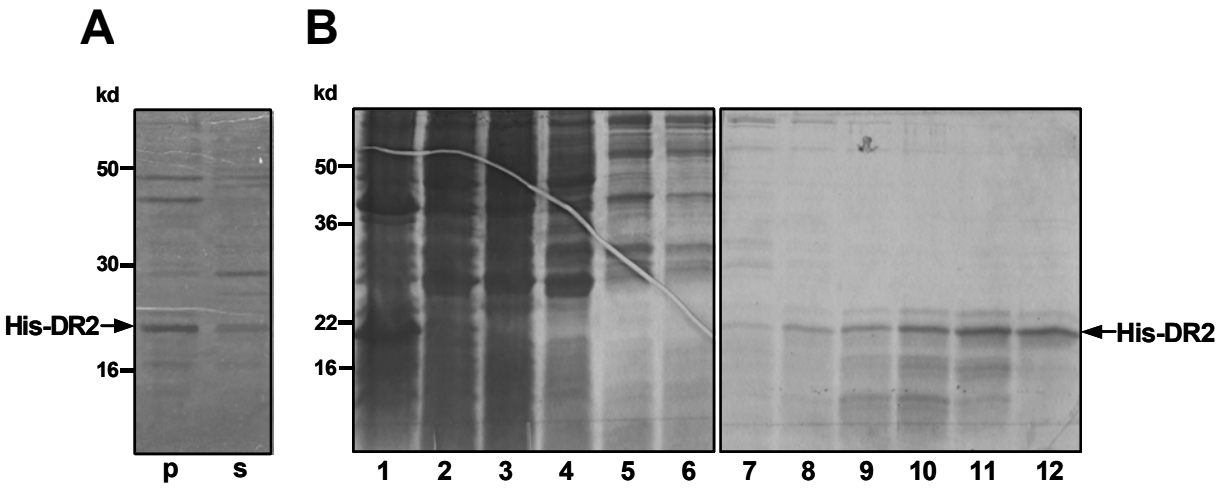
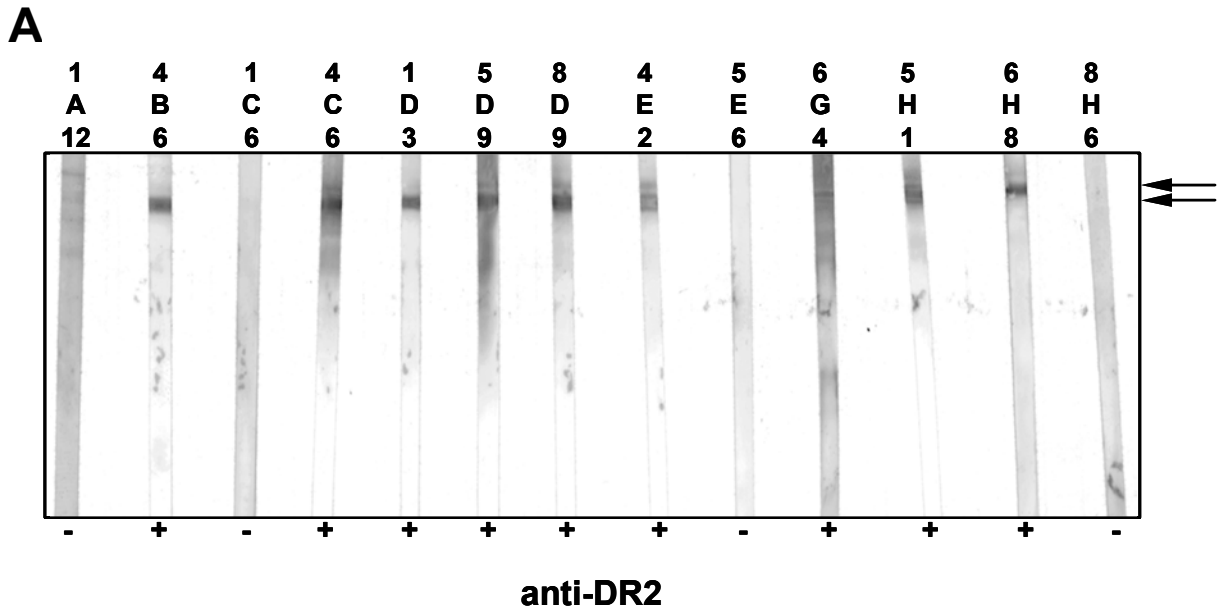


Fig. 8: Expression and purification of DR2-HisTag-fusion protein

(A) Expression of DR2-HisTag-fusion protein in *E. coli*. The DNA encoding DR2 repeats was cloned into the bacterial expression vector pET-15b and the DR2-HisTag-fusion protein was expressed as described in Materials and Methods. Samples of the resuspended pellet (p) as well as the supernatant (s) obtained after sonication were analysed by SDS-PAGE. Specific bands are indicated by arrows. (B) Elution of DR2-HisTag-fusion protein. Recombinant DR2-HisTag-fusion protein was eluted from the Ni-agarose column as described in Material and Methods. Samples of the resuspended pellet (lane 1) and the supernatant (lane 2) obtained after sonication, flow through (lane 3), washing steps (lane 4, 3 mM imidazole buffer; lane 5 and 6, 50 mM imidazole buffer) and eluted fractions (lanes 7-12, each 2x eluted with 50 mM, 100 mM and 150 mM imidazole buffer, respectively) were analysed by SDS-PAGE. Specific bands are indicated by arrows.

4.3 Generation of monoclonal antibodies against DR2 and DR1 repeat regions

To generate monoclonal rat antibodies, the purified recombinant DR proteins were used for immunization of Lou/C rats as described in Material and Methods (done in cooperation with Elisabeth Kremmer, GSF, Munich, Germany). The hybridoma supernatants containing the rat monoclonal antibodies were tested by Western Blot analysis using total cell lysate of the n-butyrate induced, HHV-8 positive cell line BCBL-1. Antibodies in several of the tested hybridoma supernatants detected bands that correlated to the predicted sizes of the respective *kaposin* isoforms (shown for DR2 only, Fig. 9A and see also below). After the subcloning of the hybridoma cells, only clones 4C6 and 6H8 (recognizing DR2) as well as clones 3C12 and 8D10 (recognizing DR1) produced still antibodies that were tested positive in different immunoassays. These antibodies showed activity in Western Blots, immunofluorescence and immunoprecipitation (Fig. 9B and Table 1, data not shown) and were used in the following experiments. The isotypes of antibodies of clones kdr1-4C6, kdr2-6H8 and kdr1-8D10 were determined to be isotype IgG_1, clone 3C12 was determined as isotype IgG_{2a}.



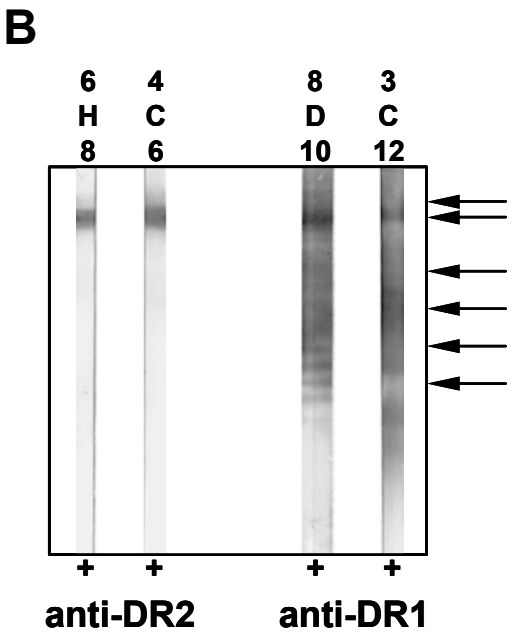


Fig. 9: Test of hybridoma supernatants by Western Blot analysis

(A) Test of hybridoma supernatants of several clones before subcloning (e.g. anti-DR2). SDS-PAGE gels of induced BCBL-1 lysates were blotted onto nitrocellulose. Subsequently, the membranes were cut into small strips. Each strip was incubated with a different hybridoma supernatant and subsequently developed as described in Material and Methods. Specific bands are indicated by arrows. (B) Test of positive hybridoma supernatants of anti-DR2 and anti-DR1 clones after subcloning. Experiments were performed similarly as described above. Specific bands are indicated by arrows.

mab	antigen	species	isotype	reactivity against HHV-8 (BCBL-1)		
				WB	IF	IP
kdr2-4C6	DR2 repeats	rat	IgG_1	+	+	+
kdr2-6H8	DR2 repeats	rat	IgG_1	+	+	+
kdr1-8D10	DR1 repeats	rat	IgG_1	+	+	+
kdr1-3C12	DR1 repeats	rat	IgG_{2a}	+	+	+

Table 1: Test of monoclonal antibodies against DR2 and DR1 repeat regions by Western blotting (WB), immunofluorescence (IF) and immunoprecipitation (IP).

4.4 A variety of *kaposin* isoforms is generated by initiation at multiple start codons

Kaposin A, the product of the originally identified KSHV ORF K12, is expressed by the majority of unstimulated PEL cells latently infected with KSHV. In contrast, antibodies against DR2 and DR1 recognize only a small number of unstimulated PEL cells, which considerably increases after stimulation with n-butyrate or phorbol ester, indicating that *kaposin* isoforms containing these repeats are expressed rather during the lytic phase (data not shown). To biochemically characterize the expression pattern of the *kaposin* protein isoforms in KSHV-infected cells, lysates of the n-butyrate-induced PEL cell line BCBL-1 were analysed by SDS-PAGE and Western blot analysis using monoclonal antibodies against single protein domains (Fig. 10). Using antibodies against DR2, two bands were detected. The faint band of approximately 54 kd correlates with the size of the translational product initiating from a CUG codon 5' of the DR2 repeats in frame 1 and represents the *kaposin C* isoform. The strongly predominant second band of about 48 kd representing the *kaposin B* isoform correlates with the size of a translation product that initiates at the first CUG codon in reading frame 2. As presumed, these two bands representing *kaposin B* and *C* were also detected by an antibody against DR1. Additionally, a whole array of bands ranging from approximately 38 to 24 kd (after longer exposure down to 18 kd) were detected, which represents isoforms that initiate from CUGs within each DR1 repeat in all three reading frames. Whereas isoforms expressed from reading frames 2 and 3 cease at the end of the DR1 repeats due to stop codons, isoforms expressed from reading frame 1 continue to the stop codon of ORF K12 and are thus approximately 6 kd larger in size (Fig. 11). To discriminate these isoforms, the terms *kaposin D* for isoforms consisting of DR1 repeats and ORF K12 (reading frame 1) and *kaposin E* for isoforms consisting only of DR1 repeats (frame 2 and 3) were introduced. Since the CUG sequence context is identical in all three reading frames, initiation should occur at an equal rate and therefore *kaposin E* is expected to be two times more abundant than *kaposin D*. The antibody against K12 detected the *kaposin A, C* and *D* isoforms, but not *kaposin B* and *E*. The protein band at 12 kd rather represents a dimeric form of *kaposin A* than a small form of *kaposin D*, since it was not detected by the anti-DR1 antibody. A similar expression pattern was observed in 293 cells transfected with a plasmid expressing a XhoII/Nhe1 fragment containing the

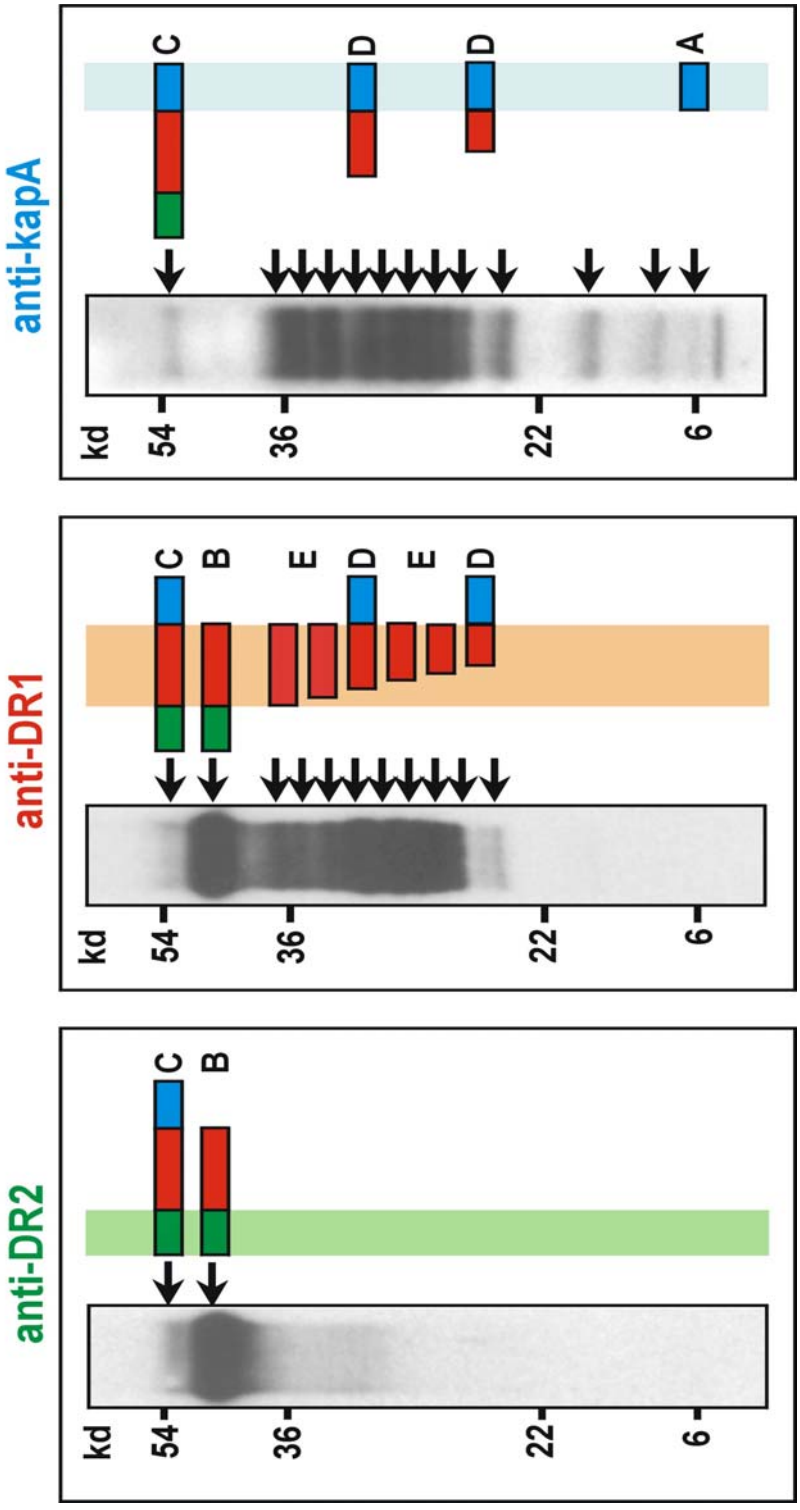


Fig. 10: Identification of *kaposin* isoforms in BCBL-1 PEL cells by Western blot analysis using mabs against single domains

Total cell lysates of n-butyrate induced BCBL-1 cells were analysed by Western blot analysis. Cells were lysed 48 h after stimulation and proteins were separated by SDS-PAGE on a 15% gel. The *kaposin* isoforms containing the DR2 repeat region were detected with a mixture of the two mabs kdr2-4C6 and kdr2-6H8, the isoforms containing the DR1 repeat region with the monoclonal antibody kdr1-8D10 and the isoforms containing the *kaposin A* region with the monoclonal antibody kap-4F11. The arrows point to distinct isoforms, which are schematically depicted. The DR2 repeat region is coloured in green, the DR1 repeat region in red and the K12 region in blue. The binding sites of the DR2, DR1 and K12 antibodies are indicated by light green, red and blue bars, respectively.

whole genomic region under control of a CMV promoter (Fig. 12, *Kaposin C*). In summary, the Western blot analyses indicate that a variety of different *kaposin* isoforms is produced from one single transcript in PEL cells, indicating that the CUGs start codons at the 5' end of DR2 and in DR1, as well as the AUG at the 5' end of K12 are used by a leaky scanning mechanism.

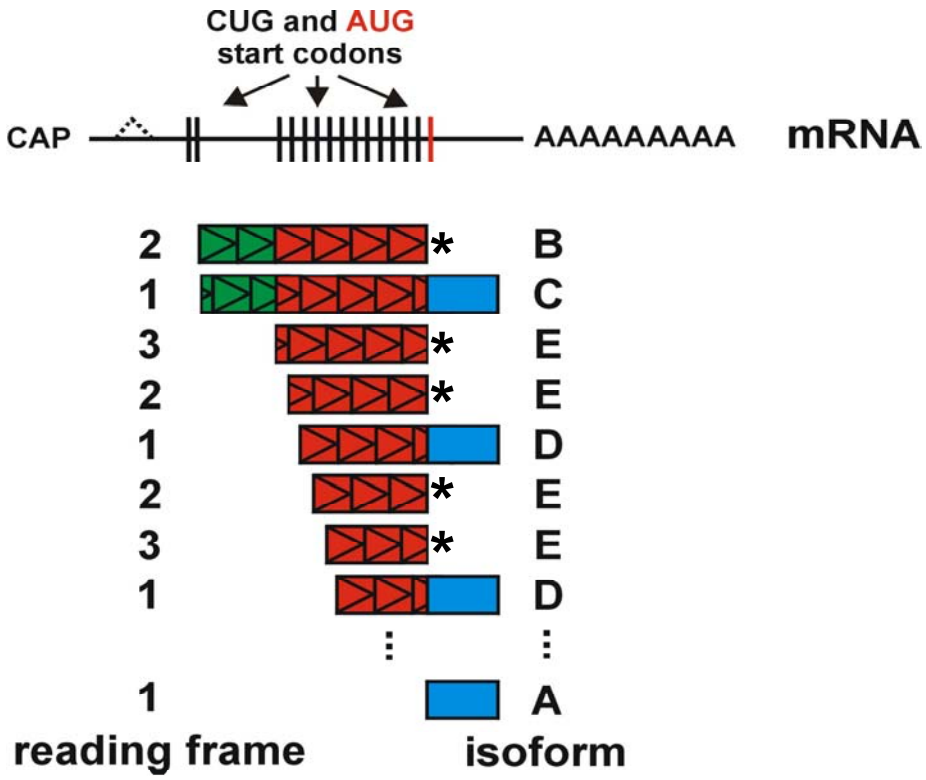


Fig. 11: *Kaposin* is expressed from multiple translational initiation sites in PEL cells

Schematic diagram of the *kaposin* protein isoforms expressed from different CUG and AUG start codons on the viral transcript. *Kaposin* protein isoforms are sorted according to the position of their start codon with the upmost isoform expressed from the first start codon on the transcript. *Kaposin* isoforms are termed according to the domains they contain: A … K12; B … DR2 + DR1; C ... DR2 + DR1 + K12; D … DR1 + K12; E … DR1. Stop codons are indicated by asterisks. *Kaposin* transcripts have been reported to be spliced 5' of DR2 in some cell lines (Li et al., 2002). Square boxes with triangles mark single DR2 or DR1 repeats.

4.5 *Kaposin* isoforms localize to different cellular compartments

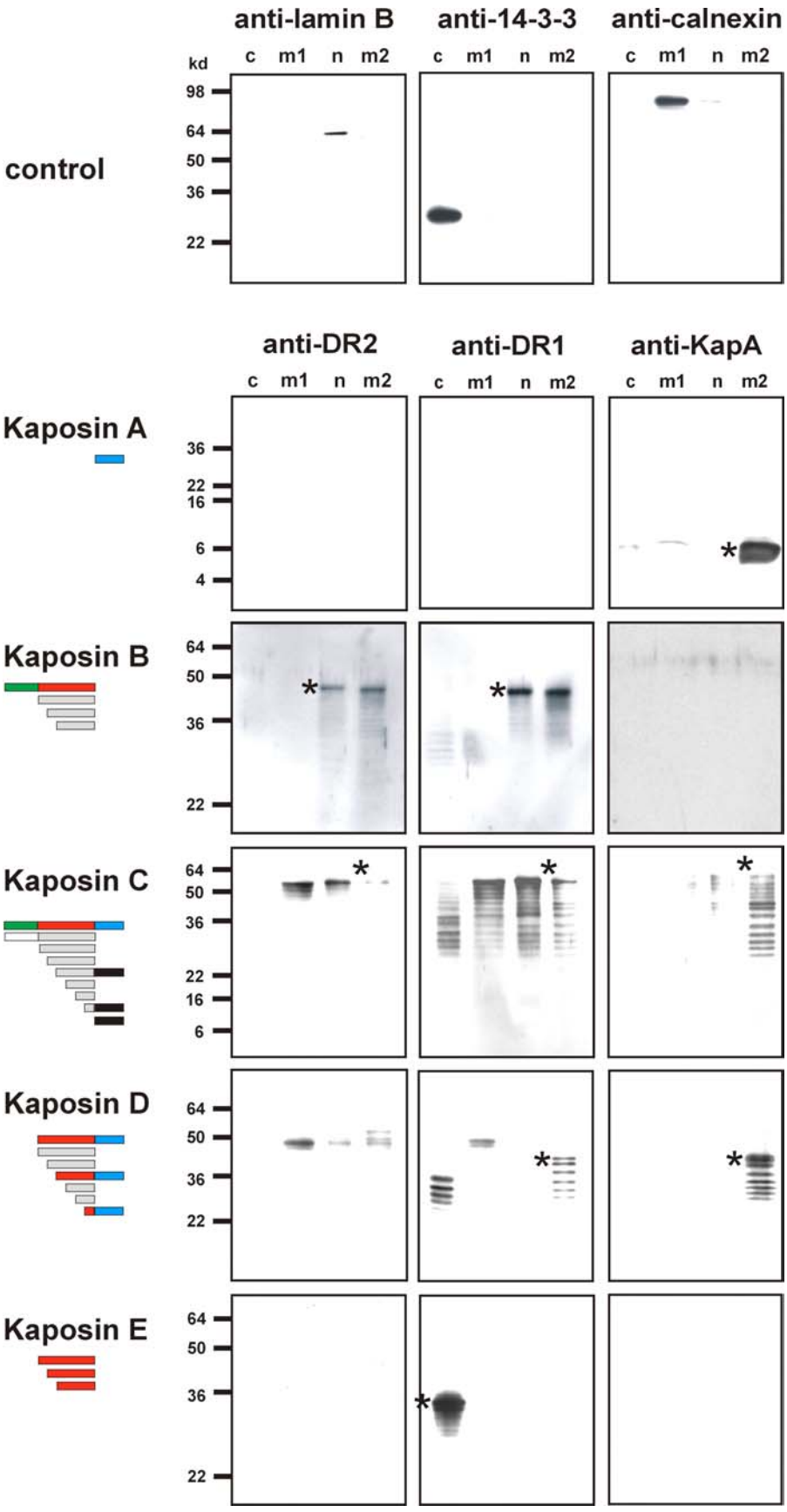
To evaluate the localization of the *kaposin* isoforms within the cell, constructs expressing single or few *kaposin* isoforms were generated and tested by fractionation (Fig. 12) and immunofluorescence after transfection (Fig. 13). Cellular subfractions of transiently transfected 293 cells were analysed by Western blot analysis with mabs against each of the three domains (Fig. 12). As reported previously, *kaposin A* was detected nearly exclusively in the unsoluble membrane fraction (m2) in the fractionation experiment (Kliche et al., 2001; Muralidhar et al., 1998; Muralidhar et al., 2000). *Kaposin B* was predominantly located in the nuclear fraction and in the unsoluble membrane fraction (presumably because of a contamination with nucleic components). In accordance with the K12 transmembrane domain, the *kaposin C* and *D* isoforms were found exclusively in the unsoluble membrane fraction (m2). In contrast, *kaposin E* was detected solely in the cytosolic fraction and thus represents a cytosolic member of the *kaposin* protein family. The difference in size between *kaposin D* and *kaposin E* corresponds to the 6 kd size of ORF K12. Due to the occurrence of alternative start codons in DR1, *kaposin E* is co-expressed with *kaposin B, C* and *D*, and *kaposin B, D* and *E* with *kaposin C*. The localization in distinct cellular compartments was confirmed by immunofluorescence in transfected Hela cells (Fig. 13). *Kaposin A*, *C* and *D* were located in vesicular structures and at the plasma membrane, *kaposin B* in the nucleus and *kaposin E* in the cytosol.

The *kaposin D* construct used in these experiments still contained the DR2 repeat region but without the upstream CUGs, since it was not expressed if DR2 was completely deleted. Thus, *kaposin B* and *C* expression was reduced but not completely abolished and some nuclear (*kaposin B*) and membrane-associated (*kaposin C*) localization was still detected with the DR2 antibody.

Fig. 12: Cellular fractionation reveals a distinct subcellular localization pattern of different *kaposin* isoforms in transfected 293 cells

Plasmid constructs expressing distinct *kaposin* isoforms (pCR3kapB, pBCBL-1, pCR3kapD, pCR3kapE) were transiently transfected into 293 cells and subcellular fractions were analysed by Western blot analysis using antibodies against DR2, DR1 and K12. *Kaposin A* was expressed by a recombinant vaccinia virus. Subcellular fractions (c: cytosolic fraction; m1: detergent-soluble membrane fraction; n: nuclear fraction; m2: detergent-unsoluble membrane fraction) were separated by SDS-PAGE on 12% gels (*kaposin A* 20%), blotted and stained with anti-DR2, anti-DR1 or anti-K12 mabs. The scheme indicates the isoforms expressed by each construct. The asterisks indicate the corresponding band(s) for each isoform. The purity of the nuclear, cytosolic and detergent-soluble membrane fractions was controlled by the marker proteins lamin B, 14-3-3 and calnexin, respectively. The *kaposin D* construct used in this experiment still contained the DR2 repeat region but without

CUGs, since it was not expressed if DR2 was completely deleted. This is the reason why some material is still detected in the m1, m2 and n fractions recognized with the anti-DR2 mab.



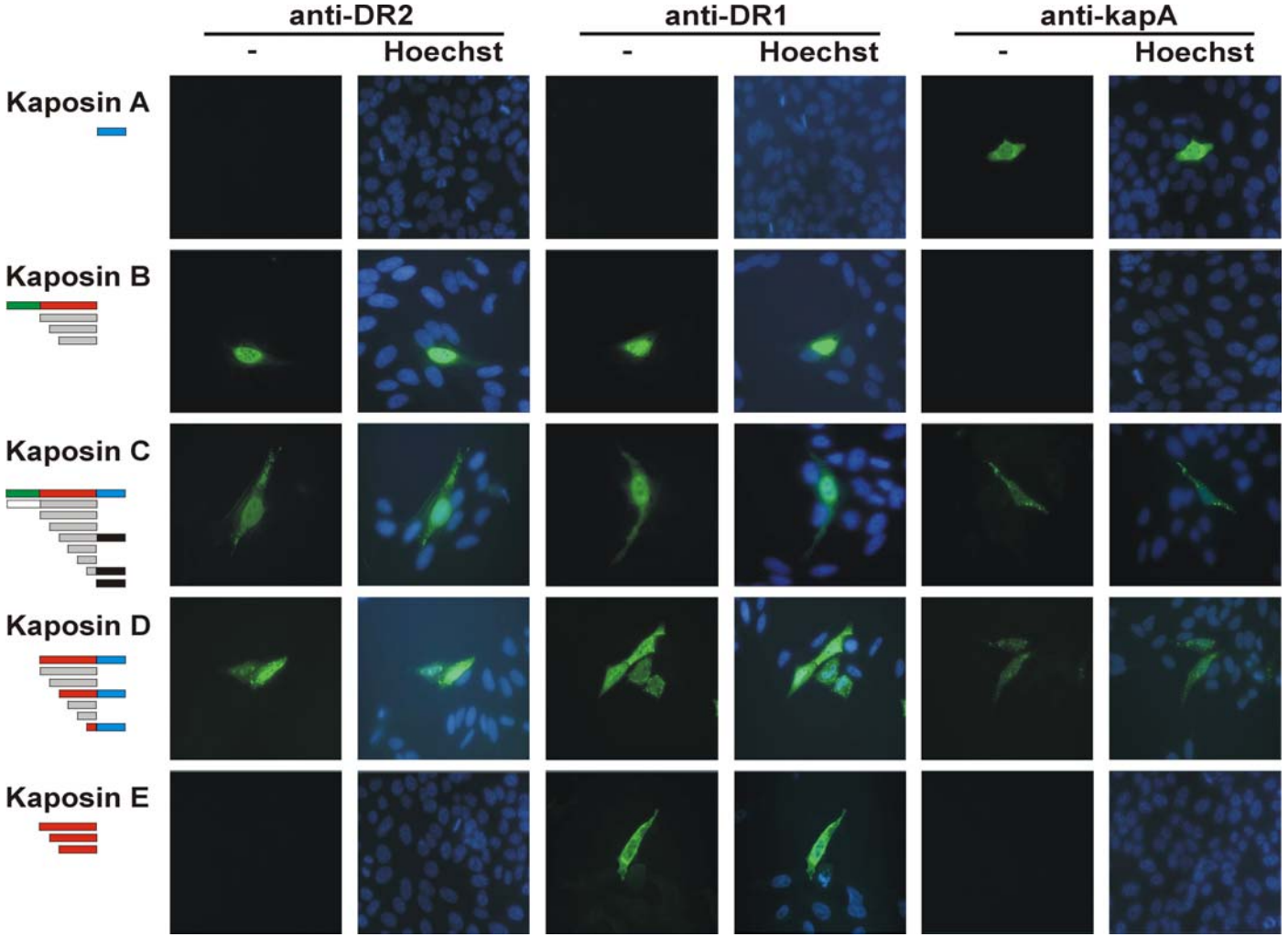


Fig. 13: Immunofluorescence analysis indicates a distinct subcellular localization pattern of *kaposin* isoforms

Immunofluorescence analysis in Hela cells transiently transfected with plasmid constructs expressing distinct *kaposin* isoforms. Hela cells were transfected with plasmids encoding for the different *kaposin* isoforms (pCR3Ig0.2, pCR3kapB, pBCBL-1, pCR3kapD, pCR3kapE). Twentyfour hours post transfection, cells were methanol-fixed and stained with either anti-DR2, anti-DR1 or anti-*kaposin A* mabs. Subsequently, cells were reacted with a secondary FITC-conjugated anti-rat antibody and Hoechst dye counterstaining nuclear DNA. The *kaposin D* construct used in this experiment still contained DR2 but without CUG start codons. Thus, *kaposin B* and *C* expression was reduced but not completely abolished and some nuclear (*kaposin B*) and vesicular staining (*kaposin C*) was still detected with the DR2 antibody.

To confirm subcellular distribution in naturally infected PEL cells, n-butyrate induced KSHV+ BCBL-1 cells were analysed by double immunfluorescence staining and cellular fractionation.

For the double immunofluorescence staining, the induced BCBL-1 cells were co-stained with different combinations of antibodies (Fig. 14). Detection with an anti-DR2 mab showed a predominant nuclear, as well as some vesicular staining. The anti-DR1 mab showed also nuclear staining but recognized additionally cytoplasmatic structures. The anti-*kaposin A* mab detected extended membrane-associated staining (Fig. 14 middle panel) and also vesicular structures, which are located in the perinuclear area of the cell (Fig. 14 lower panel). In the merge of anti-DR2 and anti-DR1, the localization of the *kaposin* isoforms *B* (yellow nuclear staining) and *C* (yellow vesicular staining) can be distinguished from the isoforms *D* and *E* (green cytoplasmatic staining) (Fig. 14 upper panel).

In parallel to the immunofluorescence analysis, cellular fractionation of induced BCBL-1 cells was performed (Fig. 15). In principle, this experiment displays a two-dimensional resolution of the Western blot analysis of total cell lysate shown before (see Fig. 10). The evaluation of the Western blot analysis of the subcellular fractions showed similar results as seen with transfected cells (compare Fig. 12): *Kaposin B* was predominantly located in the nuclear subfraction, wheras *kaposin C* was found exclusively in the detergent unsoluble membrane fraction. The likewise membrane-associated *kaposin D* isoforms differed around 6kd (the size of the ORF K12) from the cytosolic *kaposin E* isoforms (Fig. 15 middle) and *kaposin A* was again shown to be located in the detergent unsoluble membrane fraction.

In summary, both experiments confirmed that in lytic reactivated BCBL-1 cells all *kaposin* isoforms are similarly localized as observed by expression in transiently transfected cells.

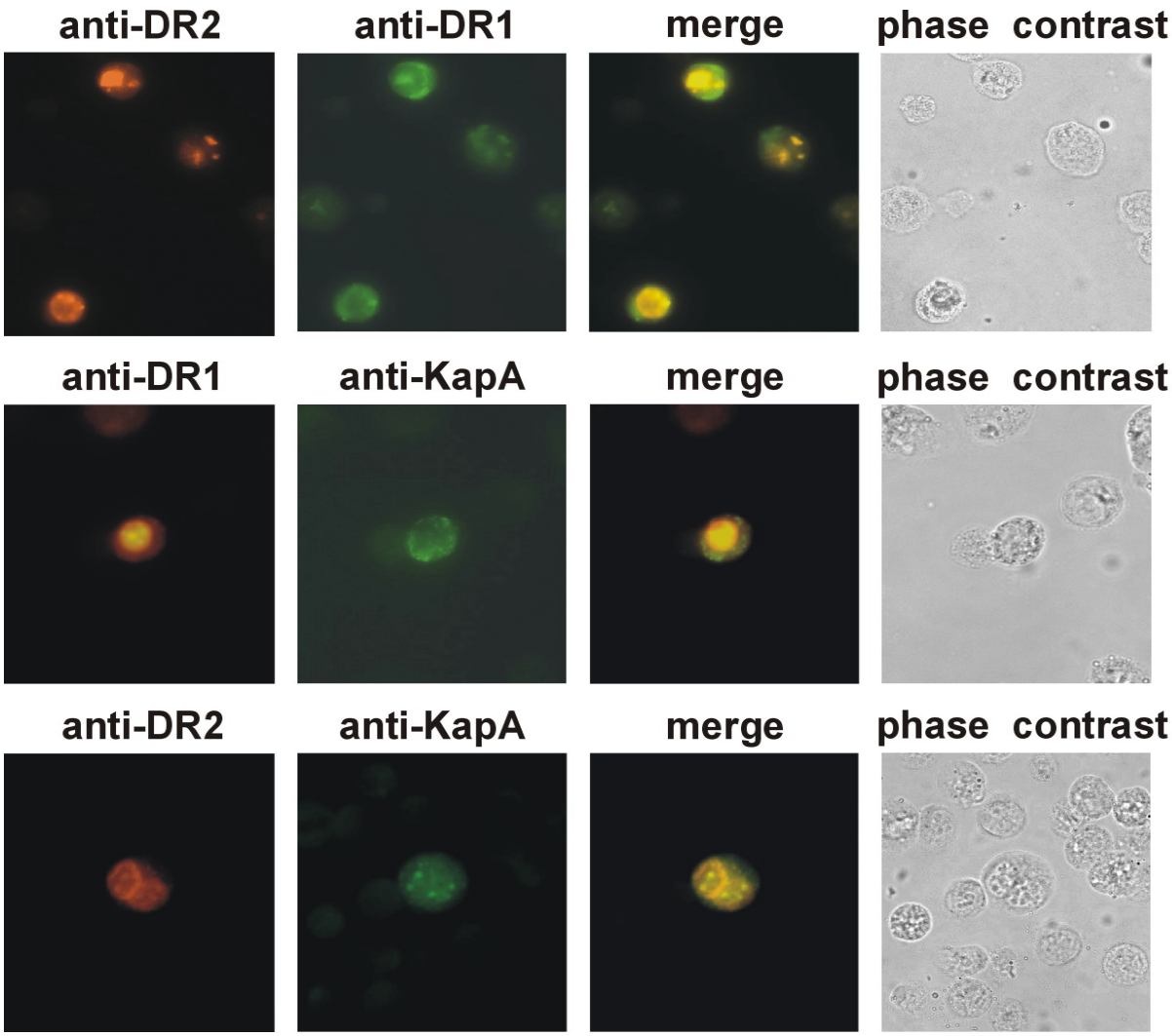


Fig. 14: Immunofluorescence analysis in BCBL-1 PEL cells

Localization of different *kaposin* isoforms in KSHV-infected cells. N-butyrate induced BCBL-1 cells were fixed with methanol and co-stained with either kdr2-4C6 (anti-DR2) and kdr1-3C12 (anti-DR1) (top), kdr1-8D10 (anti-DR1) and kap-4F11 (anti-K12) (middle) or kdr2-4C6 and kap-4F11(bottom). Subsequently, cells were stained with FITC- and Texas red-conjugated secondary reagents.

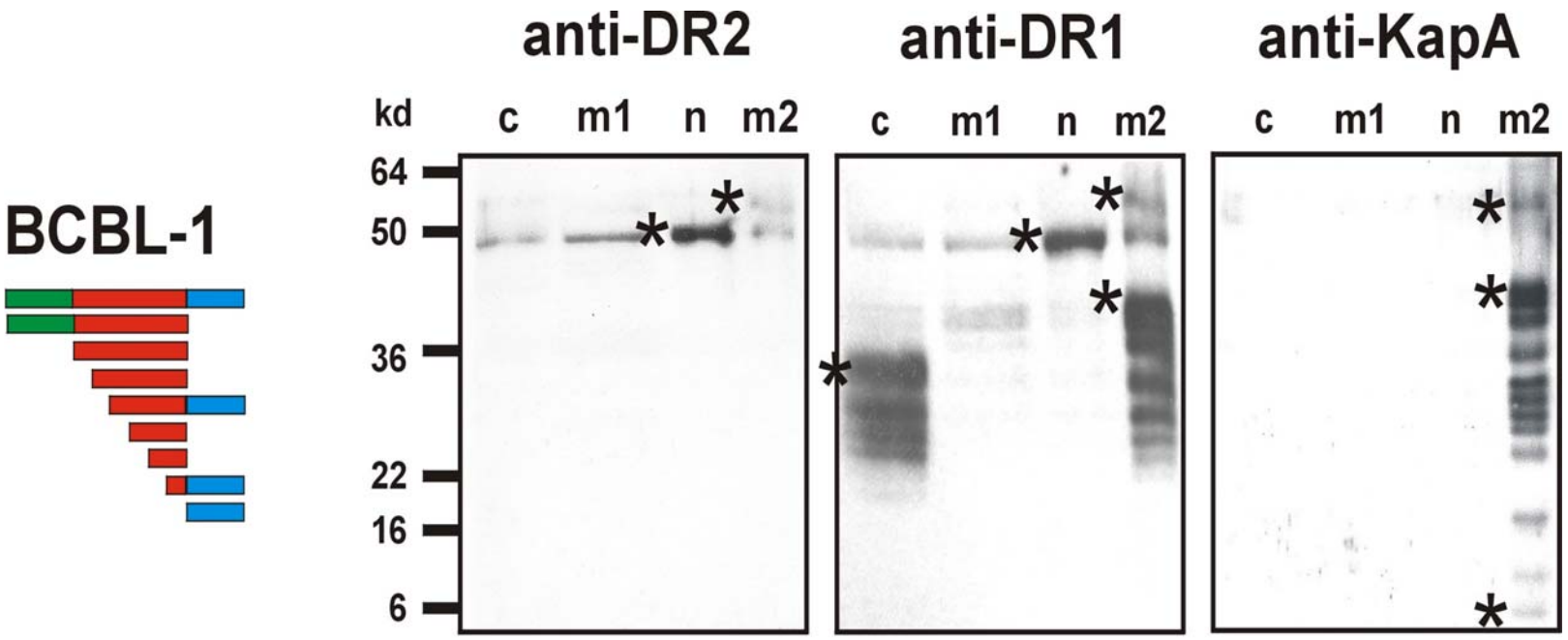


Fig. 15: Subcellular localization of different *kaposin* isoforms was confirmed in KSHV infected PEL cell line BCBL-1 by cellular fractionation

BCBL-1 cells were induced for 48 h with n-butyrate and subsequently fractionated. Subcellular fractions were analysed by Western blot analysis using antibodies against DR2, DR1 and K12. Subcellular fractions (c: cytosolic fraction; m1: detergent-soluble membrane fraction; n: nuclear fraction; m2: detergent-unsoluble membrane fraction) were separated by SDS-PAGE on 12% gels, blotted and stained with anti-DR2, anti-DR1 or anti-K12 mabs. The graphics on the left side shows schematically the isoforms expressed by induced BCBL-1 cells. The asterisks indicate the corresponding band(s) for each isoform.

4.6 *Kaposin* is a transcriptional activator

It was previously found by luciferase reporter gene assays that *kaposin A* induces the TRE promoter element containing an AP-1 binding site (Kliche et al., 2001). Thus, in this study it was tested whether *kaposin* isoforms *B, C, D* and *E*, which are expressed during the lytic phase, are also transcriptional activators. A variety of different promoters containing binding sites for AP-1, CRE, NF-κB and p53, as well as promoters with SRE and ISRE elements, HIV-1 LTR and cellular IL-6 and VEGF promoters were screened with a genomic construct expressing all *kaposin* isoforms (Fig. 16) also by performing by luciferase reporter gene assays. *Kaposin* activated AP-1 to a similar level as phorbol ester/ionomycin stimulation. *Kaposin* also slightly activated the interleukin-6 (IL-6) and VEGF-1 promoters, suggesting a putative role in the regulation of these cellular promoters. None of the other promoters was activated by *kaposin* considerably, outruling a general inductive effect.

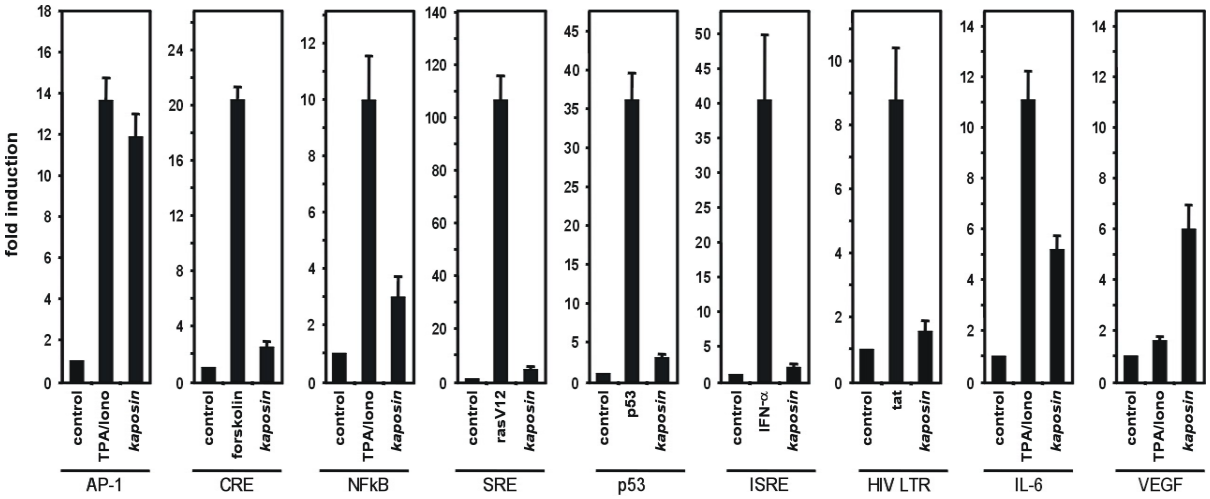


Fig. 16: *Kaposin* activates the AP-1 transcription factor and cellular promoters

An expression plasmid expressing all *kaposin* isoforms was co-transfected into 293 cells with a variety of luciferase reporter plasmids with different binding sites or promoters: AP-1, CRE, NF-κB, p53, SRE, ISRE, HIV-1 LTR, IL-6 promoter, VEGF-1 promoter. As a negative control, cells were co-transfected with pCR3. As a positive control, cells were either co-transfected with plasmids expressing either RasV12, p53 or HIV-1 Tat, or stimulated with either IFNα, forskolin or phorbol ester (TPA)/ionomycin (Iono). Data represent mean ± standard deviation of two independent experiments performed in triplicates.

4.7 DR2 repeats contain a nuclear localization signal

Since the *kaposin B* isoform containing the DR2 repeat region localizes to the nucleus (Fig. 12-15), it was hypothesized that DR2 contains a nuclear localization signal (NLS). Although the DR2 peptide sequence shows no canonical NLS, there is a high abundance of basic and hydrophilic amino acids, which are known to be present in a classical NLS (Fig. 17). To test if the NLS was in fact located within the DR2 repeats, *kaposin B* and the DR2 or DR1 repeat regions were expressed as GFP fusion proteins in Hela cells. Immunofluorescence analysis of pEGFP-kapB and pEGFP-DR2 transfected cells indicated a complete translocation of the GFP from the cytosol into the nucleus, whereas transfection with pEGFP-DR1 resulted in a similar cellular distribution of GFP as seen in control cells, transfected with the empty pEGFP vector (Fig. 18). These results confirmed that the functional NLS, responsible for the nuclear shift of the *kaposin B* isoform, is in fact localized within the DR2 repeat region.

Fig. 17: High abundance of basic amino acids in the DR2 repeat sequence

23-amino acid sequence of one DR2 repeat, shown in single letter code. Basic arginine residues are red underlayed.

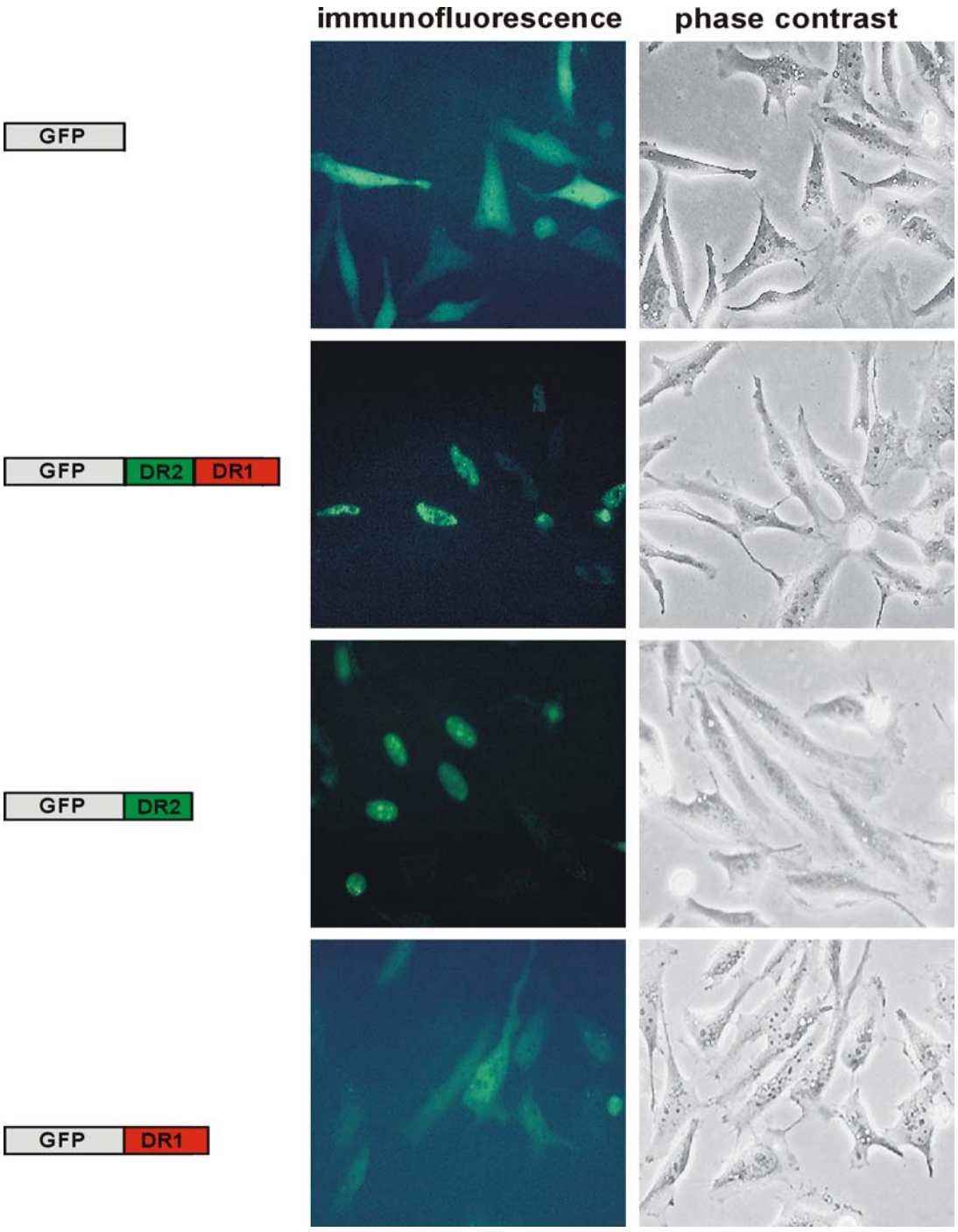


Fig. 18: The DR2 repeat region contains a functional nuclear localization signal

Hela cells were transiently transfected by calcium phosphate coprecipitation with either a pEGFP-C1 control plasmid or pEGFP-DR2, pEGFP-DR1 and pEGFP-kapB fusion constructs.

4.8 DR2 and DR1 repeat regions interact with each other

Surprisingly, when GFP fusion proteins of DR2 and DR1 were co-expressed in Hela cells, only a nuclear GFP staining was observed (Fig. 19). The cytosolic GFP-DR1 was relocalized to the nucleus by the NLS containing GFP-DR2, suggesting a direct interaction between the two repeats.

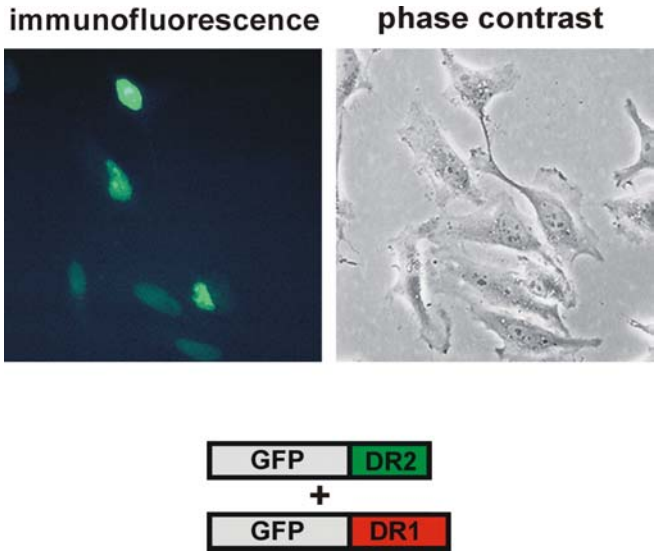


Fig. 19: Nuclear relocalization of DR1 by DR2

Hela cells were transiently transfected with pEGFP-DR2 and pEGFP-DR1 by calcium phosphate coprecipitation and subsequently analysed by immunofluorescence.

To further examine this interaction between both repeats, co-immunoprecipitation experiments were carried out. *Kaposin B*, DR2 and DR1 repeat regions were co-expressed in 293 cells as Myc- or HA-tagged proteins under the control of a T7 promoter using recombinant vaccinia virus which expresses the T7 RNA polymerase (Fuerst et al., 1986). Cell lysates were precipitated with antibodies directed against either DR2 and DR1 or HA and Myc tags. *Kaposin B* was found to interact with itself. DR1 was found to interact with itself as well as with DR2, whereas DR2 did not interact with DR2 (Fig. 20). These experiments support the results of the immunofluorescence experiment.

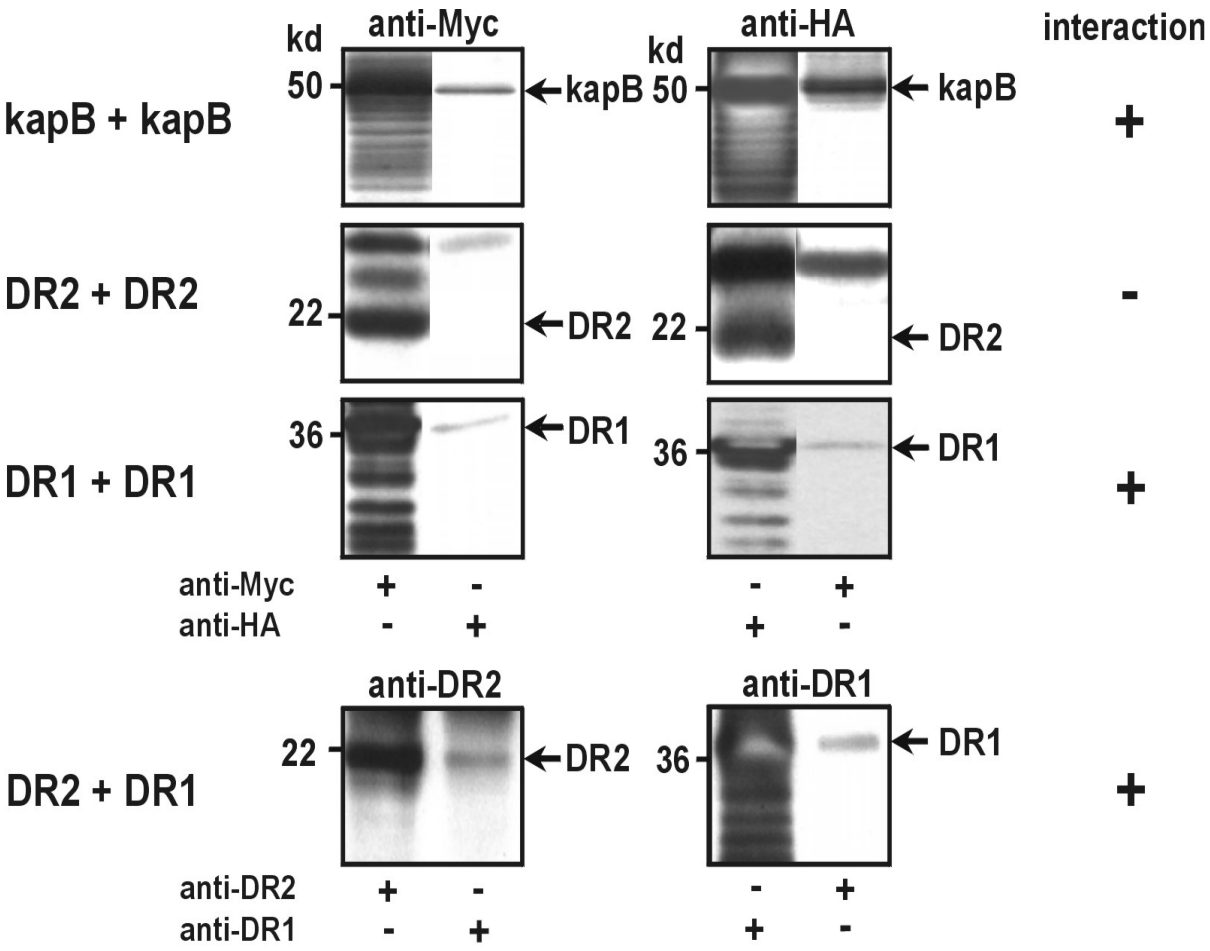


Fig. 20: DR2 and DR1 repeat regions directly interact with each other

Co-immunoprecipitation of *kaposin B*, DR2 and DR1 repeat regions. The antibodies used for the immunoprecipitation are indicated below the gels, the antibodies used for detection by Western blot above the gels and the constructs transfected on the left side of the gels. *Kaposin B* and the DR2 and DR1 repeat regions were expressed in 293 cells using pGBKT7 and pGADT7 plasmids, in which they are expressed as Myc- or HA-tagged proteins under T7 promoter control. Co-immunoprecipitation was carried out either with anti Myc- or anti HA-Tag mabs or specific mabs against DR2 and DR1.

Subsequently, the DR2 and DR1 repeat regions were tested in luciferase reporter gene assays for AP-1. Whereas isolated DR2 and DR1 repeat regions induced AP-1 only at backround levels, interestingly, co-expression of both molecules induced AP-1 approximately 7-fold compared to the negative control, further supporting a functional interaction between the two repeat regions (Fig. 21).

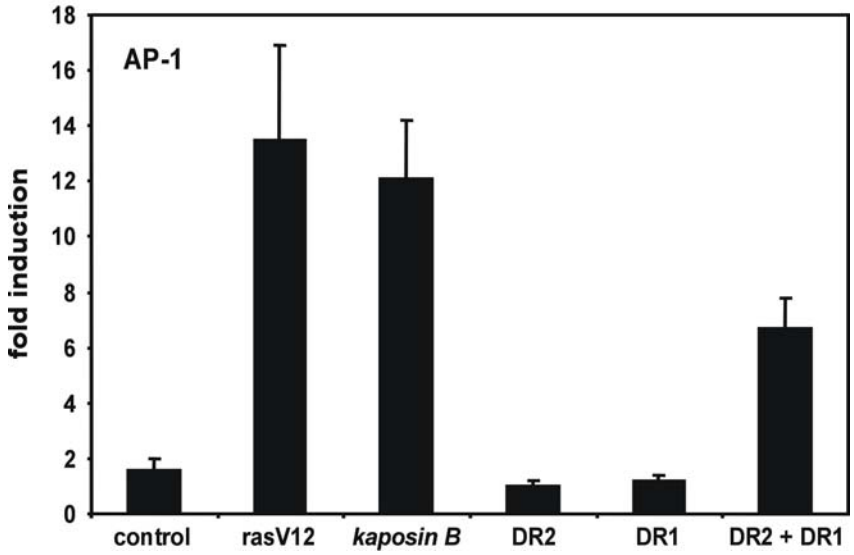


Fig. 21: Induction of AP-1 by interacting DR2 and DR1 repeats

Luciferase reporter plasmid pRTU14 (80ng), which consists of a luciferase reporter gene under the control of a minimal promoter and four TREs, was transfected into 293 cells together with 160 ng DNA of either pRK5c-mycRasV12, pEGFP-kapB, pEGFP-DR2, pEGFP-DR1 or pEGFP-DR2 and pEGFP-DR1. Negative control cells were transfected with pCR3. The control reporter plasmid pRTU1 lacking the four TRE sites showed minimal basal activity only (data not shown). Data represent mean ± standard deviation of three independent experiments performed in triplicates.

In this assay also *kaposin B* was included. The level of th AP-1 induction caused by *kaposin B* reached up to 12-fold, comparable with the positive control, the constitutively active mutant RasV12. This result indicates that the *kaposin B* isoform is responsible for the inductive effect observed with the genomic K12 construct (see Fig. 16).

4.9 Both DR2 and DR1 repeats are mandatory for AP-1 induction

To clarify if there was a correlation between AP-1 induction and nuclear localization of *kaposin B*, a construct was generated which translocates the DR1 repeats into the nucleus by a SV-40 NLS. The nuclear targeting of DR1-NLS was confirmed by immunofluorescence (Fig. 22).

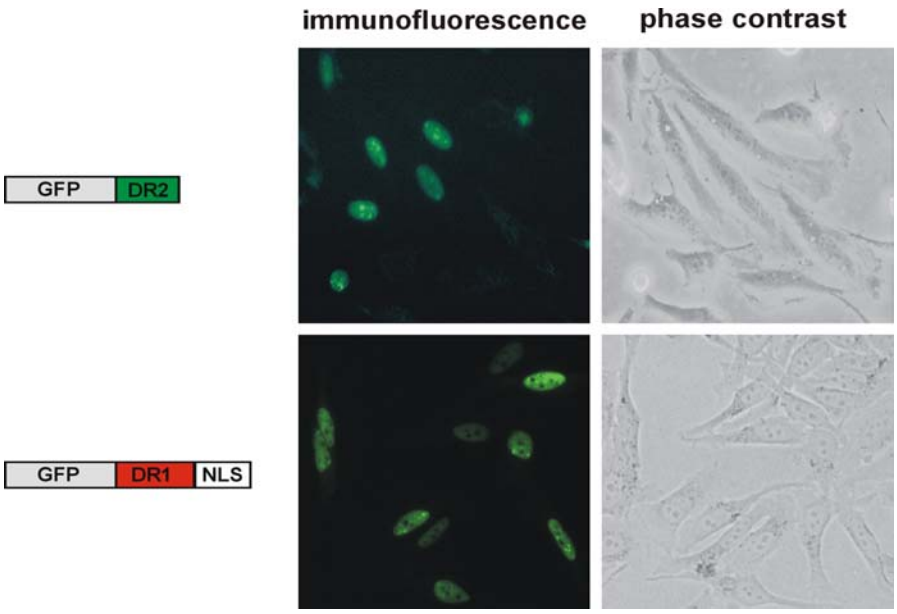


Fig. 22: DR1 repeats with a SV-40 NLS are localized in the nucleus

Immunofluorescence analysis of pEGFP-DR2 and pEGFP-DR1-NLS in transiently transfected Hela cells.

This construct was subsequently used in AP-1 luciferase gene reporter assays. Remarkably, co-expression of DR2 and DR1-NLS led to a similar activation as observed with *kaposin B* (Fig. 23). In contrast, DR2 and DR1-NLS were not able to induce AP-1 notably if expressed alone. This finding suggests that DR2 has a dual function and works not only as a targeting but also as a coactivator domain.

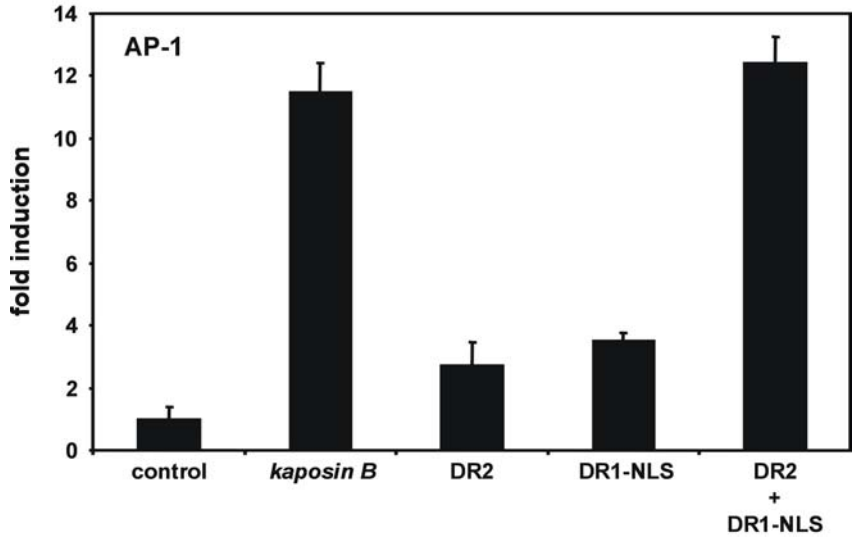


Fig. 23: Nuclear localization of both DR2 and DR1 repeat regions is mandatory for AP-1 induction

Co-expression of pEGFP-DR2 and pEGFP-DR1-NLS induces AP-1 to a similar extent as *kaposin B*. Experiments were performed similarly as described above. Data represent mean ± standard deviation of two independent experiments performed in triplicates.

4.10 Co-expression of different *kaposin* protein isoforms influences their functional activity

Since several *kaposin* isoforms are co-expressed and may bind to each other in BCBL-1 cells during the lytic phase, it was hypothesized that different isoforms functionally influence each other. To address this, individual isoforms were co-transfected into 293 cells and AP-1 luciferase assays perfomed (Fig. 24). Remarkably, *kaposin* B-induced AP-1 activation was markedly increased by co-transfection of *kaposin E*. However, this effect was counteracted by the expression of *kaposin D*. A similar inhibitory result was also achieved if *kaposin B* was co-expressed with *kaposin D*. *Kaposin D* and *E* isoforms alone, as well as in combination, were not able to induce AP-1. Thus, our data suggest that by bidirectional targeting of *kaposin E,* which contains the coactivator domain, into distinct cellular compartments through either *kaposin B* or *D*, AP-1 induction can be either increased or decreased.

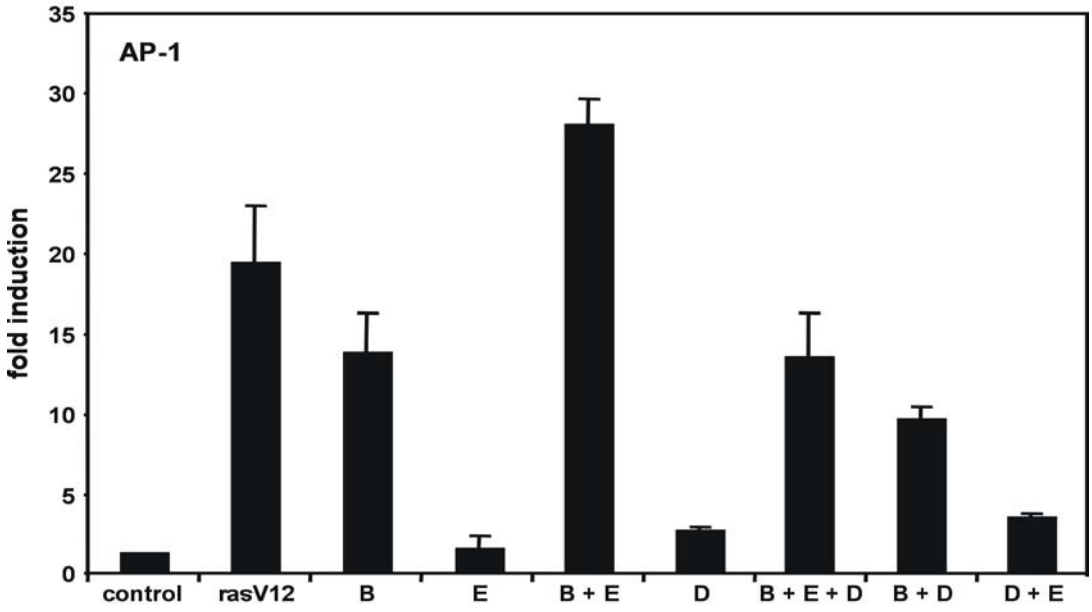


Fig. 24: Co-expression of different *kaposin* isoforms modulates their functional activity

Kaposin E and *D* modulate AP-1 activation induced by *kaposin B* in luciferase reporter assays. Equal amounts of the control vector, pRK5c-mycRasV12, pCR3kapB, pCR3kapD and pCR3kapE were transfected into 293 cells together with the AP-1 luciferase reporter plasmid pRTU-14. Similar protein amounts were used in the luciferase assays for each sample. Data represent mean ± standard deviation of two independent experiments performed in triplicates. The *kaposin D* construct used in this experiment did not contain any DR2 repeats but a HA Tag with an AUG start codon.

4.11 *Kaposin B* contains proline-rich motifs and interacts with a variety of SH3 domain proteins

Closer investigation of the repeat peptide sequences revealed that both DR2 and DR1 repeats are proline-rich and, in particular, that each repeat contains two PxxP-motifs.

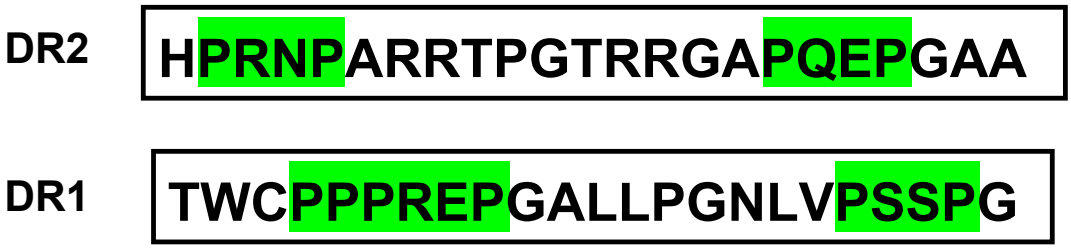


Fig. 25: Amino acid sequence of the DR2 and DR1 repeat

DR2 peptide sequence (top); DR1 peptide sequence (below). PxxP-motifs and proline-rich stretch are green underlayed.

Since PxxP motifs are known targets for proteins containing Src homology 3 (SH3) domains, several interaction partners could be possible for both repeat regions (Ren et al., 1993). SH3 domains regulate protein localization, enzymatic activity and often participate in the assembly of multicomponent signaling complexes (Mayer and Eck, 1995; Schlessinger, 1994). To test whether *kaposin* isoforms consisting of multiple of DR2 and/or DR1 repeats interact with such proteins, pull-down experiments were performed. Therefore different recombinant GST-SH3 domain proteins (provided by Dr. Stephan M. Feller, University of Oxford, Oxford, UK) were added each to NP-40-lysates of induced BCBL-1 cells and precipitated with an anti-DR1 mab bound to protein G-Sepharose. Subsequent Western blot analysis showed indeed several SH3 proteins as interaction targets, although the intensity of the interactions varied between the single domains (Fig. 26).

Considering the high abundance of proline-rich motifs in *kaposin*, the binding to SH3 domains is not surprising and the interactions seem to be more general than specific to a particular SH3 domain protein. Surprisingly, beside the n- and c-terminal SH3 domains also the SH2 domain of the Grb2 adaptor protein (molecular structure: SH3-SH2-SH3) was pulled down by *kaposin*, which can not be explained by interaction with known specific binding sites. Nevertheless, the interaction between *kaposin B*

and Grb2 was confirmed by yeast two hybrid- and co-immunoprecipitation-experiments (Table 2 and Fig. 27).

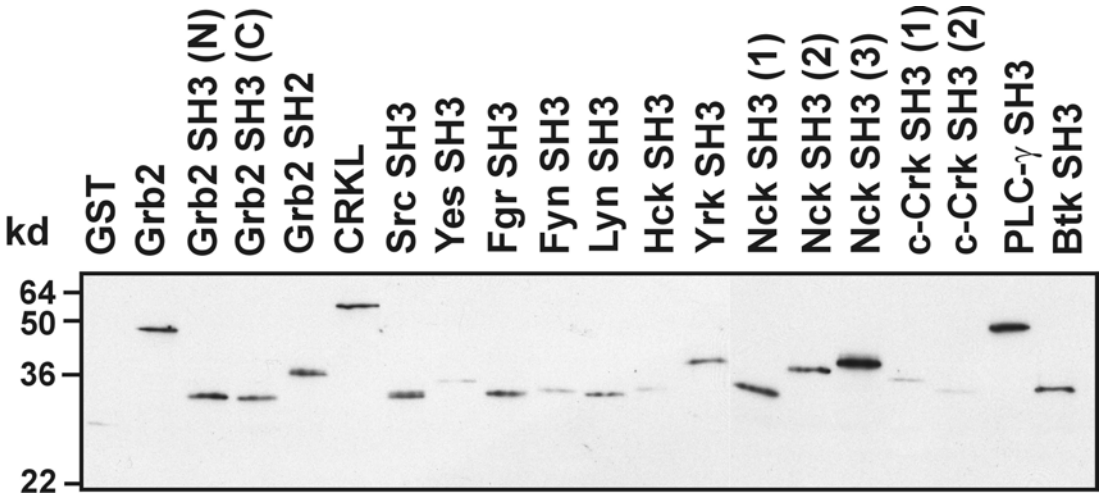


Fig. 26: *Kaposin B* interacts with different SH3 domain proteins

In a pull-down experiment NP-40 lysates of n-butyrate induced KSHV infected BCBL-1 cells were mixed with approximately 10 µg of recombinant GST-fusion proteins or GST control. Immunoprecipitation against *kaposin B* was performed as indicated in Material and Methods. After electrophoresis, proteins were blotted onto nitrocellulose and detected by an anti-GST antibody.

pGADT7 / pGBKT7	*kaposin B*	DR2	DR1
control	-	-	-
DR2	n.d.	-	-
DR1	n.d.	n.d.	false positive
Grb2	+++	(+)	-
Grb2 C-SH3	++	-	-

Table 2: Yeast two hybrid interaction test. Experiments were performed for *kaposin B*, DR2 and DR1 repeat regions, Grb2 full length (Grb2) and the c-terminal SH3 domain of Grb2 (Grb2 C-SH3) as described in Material and Methods. Since *kaposin B* and DR1 activated reporter gene expression if expressed from the bait vector pGBKT7 (containing the GAL4 DNA-binding domain) interactions with these proteins could be assayed by expressing them from the prey vector (containing the GAL4 activation domain) only (n.d.: not done). The interaction between DR2 and DR1 shown by luciferase reporter gene assay, IF and Co-IP could not be detected in this experiment.

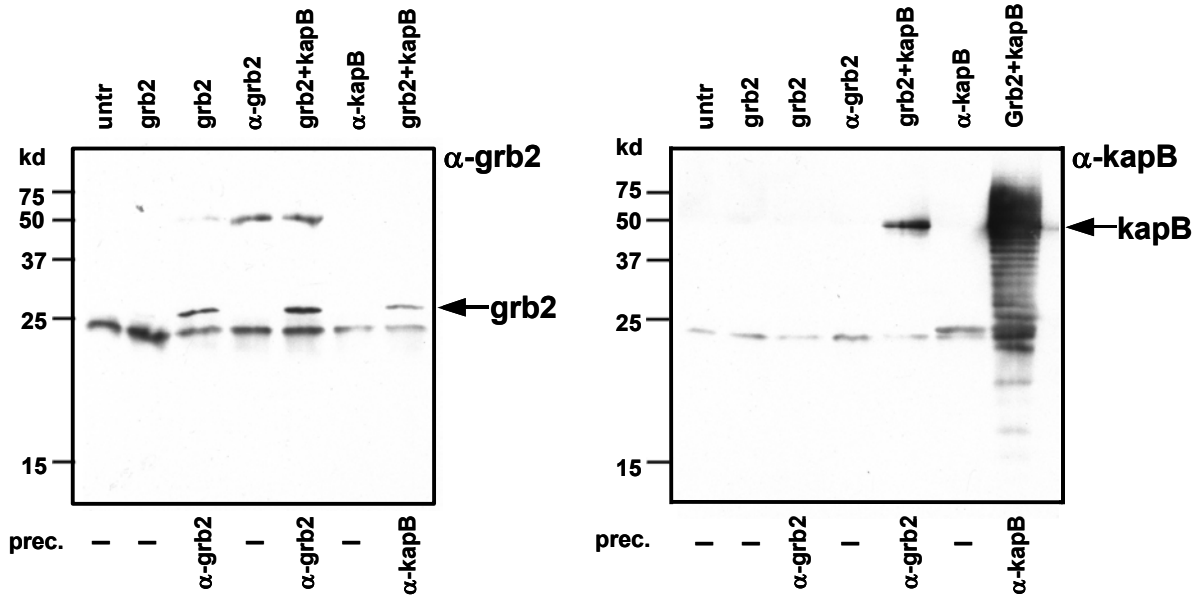


Fig. 27: The SH3 adaptor protein Grb2 interacts with *kaposin B*

Co-immunoprecipitation of *kaposin B* and full length Grb2. The antibodies used for the immunoprecipitation are indicated below the gels, antibodies used for detection by Western blot up on the right side of the gels and the constructs transfected above the gels. *Kaposin B* and Grb2 were expressed in 293 cells using pGBKT7 and pGADT7 plasmids, in which they are expressed under T7 promoter control. Specific bands are indicated by arrows.

In yeast two hybrid-experiments *kaposin B* (but not the single repeat regions) was found to interact with full length Grb2 as well as with the c-terminal SH3 domain. Interaction tests with *kaposin B* and the DR1 repeat region were tested by expressing them from the prey vector only, since both constructs showed reporter gene activation if fused to the Gal4 DNA-binding domain (Table 2).

For co-immunoprecipitation, *kaposin B* and Grb2 were co-expressed in 293 cells under the control of a T7 promoter using vaccinia virus expressing T7 RNA polymerase as described in Materials and Methods. Proteins could be co-precipitated from both sides with either anti-DR1 mabs or an anti-grb2 polyclonal antibody. Bands at approximately 27 kd (for Grb2) and 48 kd (for *kaposin B*) were detected in the cell lysates of cells transfected or co-transfected with Grb2 and *kaposin B* but not in controls (Fig. 27 left panel and right panel, respectively).

5 Discussion

Kaposi's Sarcoma Associated Herpesvirus (KSHV), which is the representative of γ2-herpesviruses in man, encodes the complex genomic *kaposin* locus consisting of two distinct sets of direct DR2 and DR1 repeats, followed by a short domain originally identified as open reading frame K12. By initiation at multiple alternative CUG (and GUG) and one single AUG start codons, a gradient of *kaposin* molecules with varying length and targeting motifs is expressed from one single transcript. In this study the expression pattern and the regulatory function of the *kaposin* locus was investigated in detail. The multitude of translational products from all three reading frames could be resolved and assigned to specific cellular compartments. Depending on the start codon used, DR1 repeats representing a functional effector domain are either fused to DR2 repeats containing a nuclear localization sequence, or to K12, which encodes a transmembrane domain. Nuclear expression of *kaposin* leads to an activation of the AP-1 transcription factor and cellular promoters. The data presented in this study indicate a novel autoregulatory mechanism based on bidirectional targeting of a viral protein to distinct subcellular compartments by expression from different start codons and reading frames. Moreover, it could be shown that certain isoforms have a mutual influence on each other.

5.1 Expression pattern and cellular localization of kaposin isoforms

In this work expression pattern and cellular localization of *kaposin* protein isoforms were examined by fractionation and immunofluorescence experiments. According to the results presented here and by others, *kaposin A* (K12) localized predominantly in tubulovesicular structures and in the detergent unsoluble membrane fraction (Muralidhar et al., 1998; Muralidhar et al., 2000; Kliche et al., 2001). Other investigators have been in doubt whether the K12 ORF is expressed at all, since internal initiation from the K12 ATG codon and extended ribosomal scanning (see below) of the 2.3 kb sized K12 transcript would be necessary (Sadler et al., 1999). In Fig. 10 (right panel), mAb 4F11 directed against the K12 ORF recognizes a band at 6 kd corresponding to the predicted 60-amino acid product of K12 ORF, demonstrating that, in fact, *kaposin A* is expressed in BCBL-1 cells *in vivo*. In contrast to the

membrane association of *kaposin A*, *kaposin B*, which is the predominant BCBL-1 K12 protein product, was found almost exclusively in the nucleus. The NLS responsible for this nuclear localization, was restricted to the DR2 repeats, which are very hydrophilic and harbor multiple basic amino acids, as it is known for other NLS. Since *kaposin C* fuses the DR2/DR1 repeat region and the hydrophobic C-terminal K12 ORF, this isoform was found to be membrane associated as hypothesized by Sadler and colleagues (Sadler et al., 1999). Furthermore, two additional groups of gene products could be separated. These isoforms initiate from multiple alternative CUG start codons within the DR1 repeats in frame 1 (representing DR1-K12 ORF fusion proteins) or in frame 2 and 3 (consisting of DR1 only). For better discrimination, the terms *kaposin D* (DR1-K12 ORF) and *kaposin E* (DR1) were proposed for these polypeptides. According to their structure, these two isoforms localize in different cellular compartments. As shown in Fig. 12 and Fig. 15, *kaposin D* localizes in the unsoluble membrane fraction, wheras *kaposin E* is exclusively found in the cytosol. The difference in size between the two arrays of *kaposin D* and *kaposin E* corresponds to the 6 kd size of the K12 ORF.

5.2 Kaposin expression and leaky scanning

The present work presents strong evidence that KSHV expresses this variety of different *kaposin* isoforms by multiple translational initiation sites rather than differential splicing. Multiple translational initiation sites occur in two different situations, either reinitiation or leaky scanning. Both are mediated through complex mRNA secondary structures which have previously been shown to occur in a variety of different cellular and viral transcripts (Bos et al., 1981; Cao and Geballe, 1995; Chen et al., 2001; Jang et al., 1988). In KSHV, the latent v-FLIP, v-cyc and LANA-1 genes are expressed from a polycistronic mRNA containing an IRES (Dittmer et al., 1998; Low et al., 2001; Sarid et al., 1999). Currently it is thought that the number of genes expressed by reinitiation and leaky scanning is limited to two or three due to a strongly decreasing efficiency (Kozak, 2002). In case of *kaposin*, a multiplicity of proteins depending on the varying number of DR1 repeats is expressed in tumors and PEL cell lines. *Kaposin* thus represents the first case in which multiple initiation sites generate an array of 15 and more different protein isoforms.

Reinitiation occurs at polycistronic mRNAs which contain more than one, usually non-overlapping ORFs (Kozak, 2002). One or more short upstream ORFs are followed by a major, longer downstream ORF. The upstream ORF(s) have regulatory function as they reduce translational initiation at the major ORF.

Leaky scanning is a mechanism that allows initation to occur at an upstream non-AUG codon in addition to the first AUG of a transcript. It is hypothesized that GC-rich mRNA sequences form secondary structures which retard scanning and thus allow mismatched Met-tRNAi to bind to the upstream non-AUG start codon (Kozak, 1991). The *kaposin* mRNA reveals a similar structure, since CUG start codons precede a downstream AUG. Interestingly, the downstream AUG and the upstream CUG start codons are used during different phases of infection. Scanning appears to be maximally leaky during the latent phase, when only the latent *kaposin A* is expressed through a downstream AUG start codon, and minimally leaky during the lytic phase, when *kaposin B*, *C*, *D* and *E* isoforms are expressed from upstream CUGs (Fig. 28).

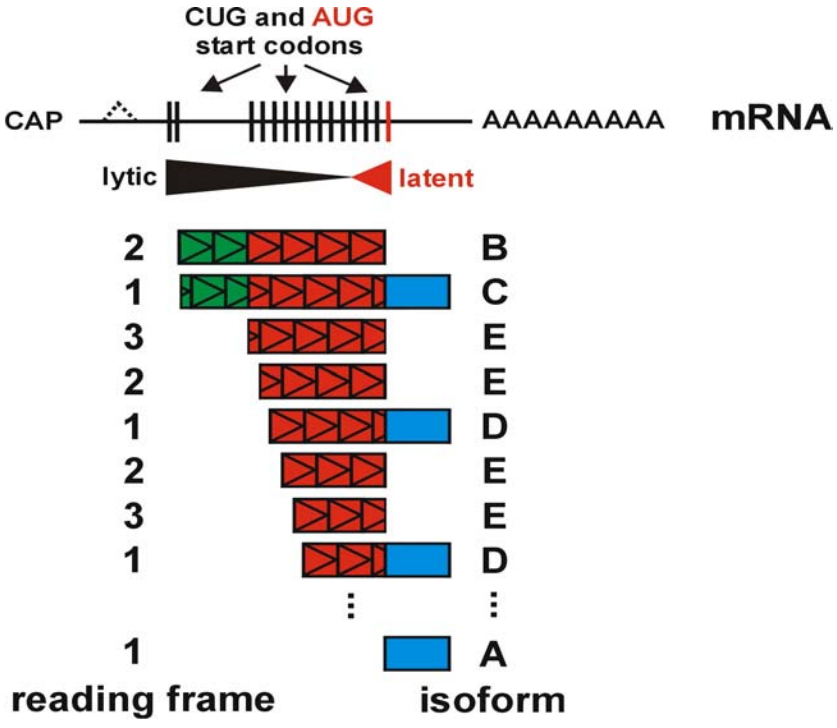


Fig. 28: Ribosomal scanning is maximally leaky during the latent phase and minimally leaky during the lytic phase

Schematic diagram of the *kaposin* protein isoforms expressed from different CUG and AUG start codons on the viral transcript (also compare Fig. 11). The model hypothesizes a translational initiation gradient from downstream to upstream start codons during the course of infection. Black and red triangles below the mRNA show the hypothesized start codon usage during latent and lytic phase.

This gives rise to the hypothesis that a gradient of translational initiation from downstream to upstream DR1 CUGs occurs during the course of infection. During the early lytic phase, downstream DR1 CUGs (near the K12 AUG which is used during the latent phase) generate short *kaposin D* and *E* isoforms which are less active. During the late lytic phase, upstream DR1 CUGs generate long *kaposin D* and *E* isoforms (as well as *kaposin B* and *C)* which are functionally more active in the presence of *kaposin B*.

Leaky scanning is a well-known phenomenon in cellular and viral transcripts and can generate long and short protein isoforms which are targeted to different cellular compartments (Kwiatkowski et al., 1988; Oda et al., 1990; Holbrook and Danpure, 2002). KSHV has adopted this cellular mechanism, however considerably refined. In KSHV, a coactivator domain is targeted to three different compartments, nucleus (*kaposin B*), cytosol (*kaposin E*) and vesicular or plasma membrane (*kaposin C* and *D*). The identical peptide sequence in all three reading frames allows KSHV to express a constant ratio of *kaposin D* and *E* independently of whether upstream or downstream CUGs are used (Fig. 28). Leaky scanning might be a regulated process with other cellular or viral proteins involved. In *S. cerevisiae*, translational reinitiation of cellular transcripts at the GCN4 locus is known to be regulated by eIF-2, which is phosphorylated and inactivated under starvation (Hinnebusch, 1993). A cellular regulator which (i) changes its expression level during the course of infection or (ii) is present at different levels in B lymphocytes and endothelial cells might be involved in the regulation of the *kaposin* isoforms. Alternatively, a viral factor could bind and stabilize the secondary structure of the mRNA sequence responsible for leaky scanning.

It was previously shown that leaky scanning is caused by highly structured GC-rich mRNA leader sequences (Kozak, 1991). The DR2 and DR1 repeat regions comply well with this prerequisite due to their complex repeat character and high GC content. In fact, there is experimental evidence that RNA secondary structure might be involved in initation at downstream CUGs since it was not possible to express *kaposin D* without an artificial AUG upstream of DR1. However, good expression of *kaposin D* and *E* was observed if DR2 was present 5' of DR1. Hence, DR2 appears to facilitate translation initiation at CUGs within DR1, probably due to a complex mRNA secondary structure. In line with this suggested function, the presence of DR2 is highly conserved in all KSHV subtypes, even in a recently reported PEL tumor that

is not expressing *kaposin B* due to the lack of a CUG start codon 5' of DR2 (Li et al., 2002).

5.3 Kaposin B mediated AP-1 induction is dependent on nuclear localization of the repeats

Although multiple functions are known for *kaposin A*, so far no functional data have been reported for other *kaposin* isoforms (Kliche et al., 2001; Muralidhar et al., 1998). This study shows that *kaposin B* induces the transcription factor AP-1. This activation depends on a nuclear localization of DR2 and DR1 repeats, as *kaposin D* and *E* isoforms which are found in the cytoplasma failed to activate AP-1. Since nuclear translocation of DR1 by adding a SV-40 NLS was not sufficient to restore activation, DR2 not simply targets DR1 to the site of action, but also acts as a coactivator (Fig. 23). AP-1 activation might play a role in cytokine induction, which is known to play an important role in the pathogenesis of KSHV. Recently, it was shown that AP-1 is induced by LANA-1 and upregulates IL-6 (An et al., 2002). In the present study, a slight upregulation of the IL-6 as well as the VEGF promoter was also observed, suggesting that *kaposin* might be involved in cytokine regulation (Fig. 16). However, due to its complex regulation *kaposin* might have other functions in parallel, similar to LANA-1, which is involved in tethering the genome to the host chromosome, viral replication, as well as in transcriptionally modulating viral and cellular genes. Currently the molecular mechanism how nuclear DR2 and DR1 repeats activate AP-1 is not known.

5.4 Interaction partners of kaposin

Both DR2 and DR1 are proline-rich and contain two PxxP-motifs each (Fig. 29). Since PxxP motifs are known targets for proteins containing SH3 domains, there are several interaction partners possible for both domains. In this work, pull-down experiments and subsequent Western blot analysis showed indeed several SH3 proteins as interaction targets of *kaposin*. Taken in account that *kaposin* isoforms consist of several DR2 and DR1 repeats, the high affinity to SH3 domains is not

surprising and it remains open if the interactions are more general than specific to a particular SH3 domain protein. SH3 domains are known to regulate protein localization, enzymatic activity and often participate in the assembly of multicomponent signaling complexes (Schlessinger, 1994; Mayer and Eck, 1995). Thus, interaction of one or more *kaposin* isoforms with these proteins support the hypothesis that *kaposins* are powerful regulatory molecules. Additionally to the PxxP-motifs, DR2 contains a putative WW domain, which is also known to bind proline-rich peptides (Fig. 29). It might be possible that the interaction between DR2 and DR1 is based on a WW domain structure, although DR2 does not contain the WW consensus binding motif PPxY and PPLP. It is also possible that DR2 intramolecularly interact with DR1, but currently there is no evidence for this.

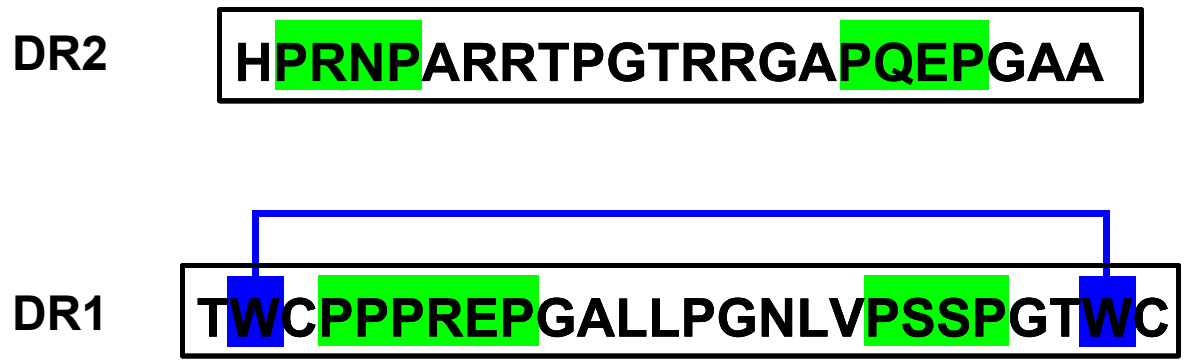


Fig. 29: Amino acid sequence of the DR2 and DR1 repeat

DR2 peptide sequence (top); DR1 peptide sequence (below). PxxP-motifs and proline-rich stretch are green underlayed. Tryptophans of the putative WW domain are blue underlayed and connected.

5.5 Differential targeting modulates functional activity

Epstein Barr Virus (EBV), the next KSHV homologue in man, belongs to the γ1-subfamily of herpesviruses. It uses a different strategy to generate distinct protein isoforms. The latent EBNA-1, 2 and 3 protein isoforms are generated by differential splicing. Similar to KSHV, however, EBV generates different isoforms of regulatory proteins to modulate it's function: the N-terminally abridged LMP-2B is known to regulate the signalling activity of LMP-2A. In KSHV, five different *kaposin* isoforms

are necessary to control and modulate each other as shown in this study. The DR1 coactivator domain is either expressed with a NLS in the nucleus, a transmembrane domain in vesicular cellular organelles and the plasma membrane, or as a single domain in the cytoplasma (Fig. 30). Hence a model is proposed in which there might be an equilibrium of *kaposin E* in different compartments, which is modulated by other *kaposin* isoforms. This modulation is either towards activation (*kaposin E* is targeted into the nucleus by *kaposin B*) or deactivation (*kaposin E* is targeted to vesicular and plasma membranes by *kaposin C* and *D)*.

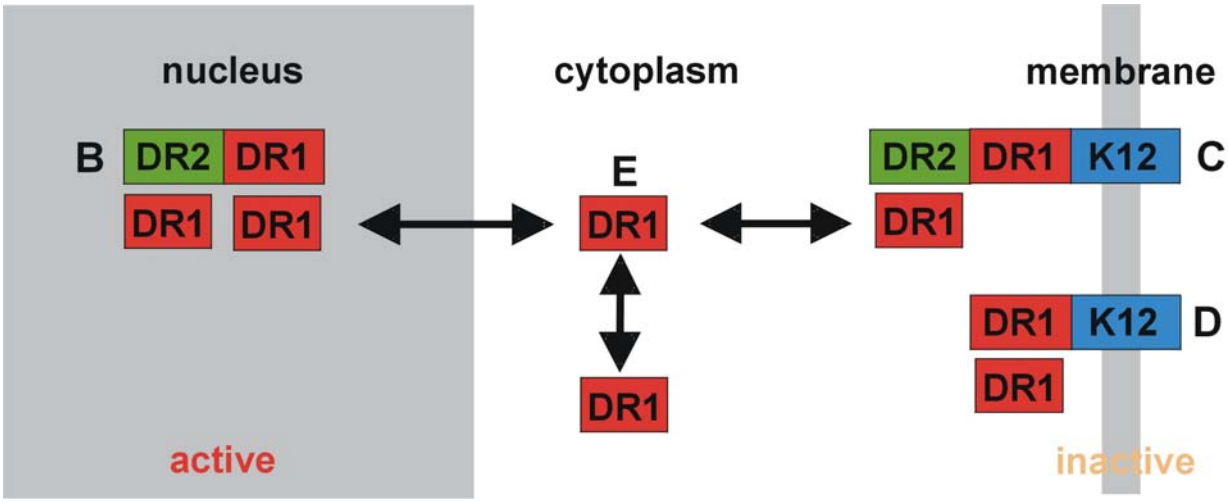


Fig. 30: Co-expression and differential targeting modulates the functional activity of different *kaposin* isoforms

Schematic diagram depicting the model by which cytosolic *kaposin E* is bidirectionally targeted either into the nucleus by *kaposin B* or to vesicular and plasma membranes by *kaposin C* and *D*.

5.6 Significance and implications

In principle, KSHV gains additional coding potential by using all three reading frames of the *kaposin* locus. However, genomic space limitation is unlikely to be the primary evolutionary selective force for expression of a particular gene in herpesviruses which have large genome sizes between 110 and 230 kb. The present study suggests that the complex expression regulation is necessary to generate a meticulous stoichiometric distribution of *kaposin* isoforms, which appears to be of critical importance for the virus. Since CUG start codons are present with an identical

sequence context in all three reading frames in DR1, *kaposin D* and *E* are expressed at a ratio of 1:2 independently of whether upstream or downstream DR1 CUGs are used (Fig. 6). More evidence for the importance of the ratio between the different *kaposin* isoforms comes from the observation that the number of the repeats varies markedly between KS tumors, but the number of DR2 and DR1 repeats is always maintained at a ratio of approximately 1:2 (Russo et al., 1996; Sadler et al., 1999). Additionally, the maintenance of this ratio may also be crucial for the proper folding or structure of the proteins. Due to the stringent regulation, *kaposin* probably plays an important role in virus pathogenicity. The complex genomic structure of *kaposin* allows the virus to *titrate* or *fine-tune* a specific viral function, probably an essential factor in the pathogenicity. During the course of infection or in a cell-type specific fashion, *kaposin* might generate a gradient of effector molecules leading to a continous switch of a specific function. In conclusion, this work presents evidence that γ2-herpesviruses developed a completely different strategy, leaky scanning, in comparison to γ1-herpesviruses, which predominantly use differential splicing, to control viral replication and cellular processes.

5.7 Perspectives

Future experiments may reveal additional functions of *kaposin* isoforms. Due to the various proline-rich binding motifs in both repeats, many interactors are possible, and the suggested switch function therefore may induce several signaling events. The prediction of those additional functions may be possible by the determination of new interactors. This question can be addressed by performing yeast two hybrid screens with *kaposin* domains. Additionally, experiments examining the kinetics of *kaposin* expression have to be carried out to investigate the proposed switch model in detail. Functional analysis is tightly linked to structural definition of *kaposins* and more structural data are necessary to confirm predictions of sequence analyses. A prerequisite for this is the purification of *kaposin* domains and isoforms, which enables the performance of spectroscopic and structural analysis. The conservation of the repeat regions and the complex translational program through practically all KSHV subtypes already strongly suggests that *kaposin* isoforms have important functions in the viral life cycle.

6 References

Ablashi,D.V., Chatlynne,L.G., and Whitman Jr.,J.E.a.C.E. (2002). Spectrum of Kaposi's sarcoma-associated herpesvirus, or human herpesvirus 8, diseases. Clin. Microbiol. Rev. *439*, 439-464.

Ahmed,A., Isa,M.S., Garba,H.A., Kalayi,G.D., Muhammad,I., and Egler,L.J. (2001). Influence of HIV infection on presentation of Kaposi's sarcoma. Trop. Doct. *31*, 42-45.

Alexander,L., Denekamp,L., Knapp,A., Auerbach,M.R., Damania,B., and Desrosiers,R.C. (2000). The primary sequence of rhesus monkey rhadinovirus isolate 26-95: sequence similarities to Kaposi's sarcoma-associated herpesvirus and rhesus monkey rhadinovirus isolate 17577. J. Virol. *74*, 3388-3398.

An,J., Lichtenstein,A.K., Brent,G., and Rettig,M.B. (2002). The Kaposi sarcoma-associated herpesvirus (KSHV) induces cellular interleukin 6 expression: role of the KSHV latency-associated nuclear antigen and the AP1 response element. Blood *99*, 649-654.

Antman,K.C.Y. (2000). Kaposi's sarcoma. N. Engl. J. Med. *342*, 1027-1038.

Arvanitakis,L., Geras,R.E., Varma,A., Gershengorn,M.C., and Cesarman,E. (1997). Human herpesvirus KSHV encodes a constitutively active G-protein-coupled receptor linked to cell proliferation. Nature *385*, 347-350.

Arvanitakis,L., Mesri,E.A., Nador,R.G., Said,J.W., Asch,A.S., Knowles,D.M., and Cesarman,E. (1996). Establishment and characterization of a primary effusion (body cavity-based) lymphoma cell line (BC-3) harboring kaposi's sarcoma-associated herpesvirus (KSHV/HHV-8) in the absence of Epstein-Barr virus. Blood *88*, 2648-2654.

AuCoin,D.P., Colletti,K.S., Xu,Y., Cei,S.A., and Pari,G.S. (2002). Kaposi's sarcoma-associated herpesvirus (human herpesvirus 8) contains two functional lytic origins of DNA replication. J. Virol. *76*, 7890-7896.

Beral,V., Peterman,T.A., Berkelman,R.L., and Jaffe,H.W. (1990). Kaposi's sarcoma among persons with AIDS: a sexually transmitted infection? Lancet *335*, 123-128.

Bos,J.L., Polder,L.J., Bernards,R., Schrier,P.I., van den Elsen,P.J., van der Eb,A.J., and van Ormondt,H. (1981). The 2.2 kb E1b mRNA of human Ad12 and Ad5 codes for two tumor antigens starting at different AUG triplets. Cell *27*, 121-131.

Boshoff,C., Endo,Y., Collins,P.D., Takeuchi,Y., Reeves,J.D., Schweickart,V.L., Siani,M.A., Sasaki,T., Williams,T.J., Gray,P.W., Moore,P.S., Chang,Y., and Weiss,R.A. (1997). Angiogenic and HIV-inhibitory functions of KSHV-encoded chemokines. Science *278*, 290-294.

Boshoff,C., Schulz,T.F., Kennedy,M.M., Graham,A.K., Fisher,C., Thomas,A., McGee,J.O., Weiss,R.A., and O'Leary,J.J. (1995). Kaposi's sarcoma-associated herpesvirus infects endothelial and spindle cells. Nat. Med. *1*, 1274-1278.

Cao,J. and Geballe,A.P. (1995). Translational inhibition by a human cytomegalovirus upstream open reading frame despite inefficient utilization of its AUG codon. J Virol *69*, 1030-1036.

Cathomas,G. (2000). Human herpes virus 8: a new virus discloses its face. Virchows Arch. *436*, 195-206.

Cerimele,F., Curreli,F., Ely,S., Friedman-Kien,A.E., Cesarman,E., and Flore,O. (2001). Kaposi's sarcoma-associated herpesvirus can productively infect primary human keratinocytes and alter their growth properties. J. Virol. *75*, 2435-2443.

Cesarman,E., Nador,R.G., Aozasa,K., Delsol,G., Said,J.W., and Knowles,D.M. (1996). Kaposi's sarcoma-associated herpesvirus in non-AIDS related lymphomas occurring in body cavities. Am. J. Pathol. *149*, 53-57.

Chan,S.R., Bloomer,C., and Chandran,B. (1998). Identification and characterization of human herpesvirus-8 lytic cycle-associated ORF 59 protein and the encoding cDNA by monoclonal antibody. Virology *240*, 118-126.

Chang,P.J., Shedd,D., Gradoville,L., Cho,M.S., Chen,L.W., Chang,J., and Miller,G. (2002). Open reading frame 50 protein of Kaposi's sarcoma-associated herpesvirus directly activates the viral PAN and K12 genes by binding to related response elements. J. Virol. *76*, 3168-3178.

Chang,Y., Cesarman,E., Pessin,M.S., Lee,F., Culpepper,J., Knowles,D.M., and Moore,P.S. (1994). Identification of herpesvirus-like DNA sequences in AIDS-associated Kaposi's sarcoma. Science *266*, 1865-1869.

Chee,M.S., Bankier,A.T., Beck,S., Bohni,R., Brown,C.M., Cerny,R., Horsnell,T., Hutchison,C.A., III, Kouzarides,T., Martignetti,J.A., and . (1990). Analysis of the protein-coding content of the sequence of human cytomegalovirus strain AD169. Curr. Top. Microbiol. Immunol. *154*, 125-169.

Chen,W., Calvo,P.A., Malide,D., Gibbs,J., Schubert,U., Bacik,I., Basta,S., O'Neill,R., Schickli,J., Palese,P., Henklein,P., Bennink,J.R., and Yewdell,J.W. (2001). A novel influenza A virus mitochondrial protein that induces cell death. Nat. Med. *7*, 1306-1312.

Ciufo,D.M., Cannon,J.S., Poole,L.J., Wu,F.Y., Murray,P., Ambinder,R.F., and Hayward,G.S. (2001). Spindle cell conversion by Kaposi's sarcoma-associated herpesvirus: formation of colonies and plaques with mixed lytic and latent gene expression in infected primary dermal microvascular endothelial cell cultures. J. Virol. *75*, 5614-5626.

Clontech (1999). Matchmaker Gal4 Two-Hybrid System 3 & Libraries User Manual. Clontech Laboratories, Inc. , Palo Alto, California, USA.

Davis,M.A., Sturzl,M.A., Blasig,C., Schreier,A., Guo,H.G., Reitz,M., Opalenik,S.R., and Browning,P.J. (1997). Expression of human herpesvirus 8-encoded cyclin D in Kaposi's sarcoma spindle cells. J. Natl. Cancer Inst. *89*, 1868-1874.

Diepgen,T.L. and Eysenbach,G. (1998). Digital images in dermatology and the Dermatology Online Atlas on the World Wide Web. J. Dermatol. *25*, 782-787.

Dittmer,D., Lagunoff,M., Renne,R., Staskus,K., Haase,A., and Ganem,D. (1998). A cluster of latently expressed genes in Kaposi's sarcoma-associated herpesvirus. J Virol *72*, 8309-8315.

Dittmer,D.P. (2003). Transcription profile of Kaposi's sarcoma-associated herpesvirus in primary Kaposi's sarcoma lesions as determined by real-time PCR arrays. Cancer Res. *63*, 2010-2015.

Dupin,N., Diss,T.L., Kellam,P., Tulliez,M., Du,M.Q., Sicard,D., Weiss,R.A., Isaacson,P.G., and Boshoff,C. (2000). HHV-8 is associated with a plasmablastic variant of Castleman disease that is linked to HHV-8-positive plasmablastic lymphoma. Blood *95*, 1406-1412.

Dupin,N., Fisher,C., Kellam,P., Ariad,S., Tulliez,M., Franck,N., van Marck,E., Salmon,D., Gorin,I., Escande,J.P., Weiss,R.A., Alitalo,K., and Boshoff,C. (1999). Distribution of human herpesvirus-8 latently infected cells in Kaposi's sarcoma, multicentric Castleman's disease, and primary effusion lymphoma. Proc. Natl. Acad. Sci. U. S. A *96*, 4546-4551.

Fakhari,F.D. and Dittmer,D.P. (2002). Charting latency transcripts in Kaposi's sarcoma-associated herpesvirus by whole-genome real-time quantitative PCR. J. Virol. *76*, 6213-6223.

Fiorelli,V., Gendelman,R., Sirianni,M.C., Chang,H.K., Colombini,S., Markham,P.D., Monini,P., Sonnabend,J., Pintus,A., Gallo,R.C., and Ensoli,B. (1998). gamma-Interferon produced by CD8+ T cells infiltrating Kaposi's sarcoma induces spindle cells with angiogenic phenotype and synergy with human immunodeficiency virus-1 Tat protein: an immune response to human herpesvirus-8 infection? Blood *91*, 956-967.

Foreman,K.E., Friborg,J., Kong,W.P., Woffendin,C., Polverini,P.J., Nickoloff,B.J., and Nabel,G.J. (1997). Propagation of a human herpesvirus from AIDS-associated Kaposi's sarcoma. N. Engl. J. Med. *336*, 163-171.

Fuerst,T.R., Niles,E.G., Studier,F.W., and Moss,B. (1986). Eukaryotic transient-expression system based on recombinant vaccinia virus that synthesizes bacteriophage T7 RNA polymerase. Proc. Natl. Acad. Sci. U. S. A *83*, 8122-8126.

Ganem,D. (1997). KSHV and Kaposi's sarcoma: the end of the beginning? Cell *91*, 157-160.

Gao,S.J., Kingsley,L., Li,M., Zheng,W., Parravicini,C., Ziegler,J., Newton,R., Rinaldo,C.R., Saah,A., Phair,J., Detels,R., Chang,Y., and Moore,P.S. (1996). KSHV antibodies among Americans, Italians and Ugandans with and without Kaposi's sarcoma. Nat. Med. *2*, 925-928.

Gill,P.S., Tsai,Y.C., Rao,A.P., Spruck,C.H., III, Zheng,T., Harrington,W.A., Jr., Cheung,T., Nathwani,B., and Jones,P.A. (1998). Evidence for multiclonality in multicentric Kaposi's sarcoma. Proc. Natl. Acad. Sci. U. S. A *95*, 8257-8261.

Godowski,P.J. and Knipe,D.M. (1986). Transcriptional control of herpesvirus gene expression: gene functions required for positive and negative regulation. Proc. Natl. Acad. Sci. U. S. A *83*, 256-260.

Gradoville,L., Gerlach,J., Grogan,E., Shedd,D., Nikiforow,S., Metroka,C., and Miller,G. (2000). Kaposi's sarcoma-associated herpesvirus open reading frame 50/Rta protein activates the entire viral lytic cycle in the HH-B2 primary effusion lymphoma cell line. J. Virol. *74*, 6207-6212.

Grandadam,M., Dupin,N., Calvez,V., Gorin,I., Blum,L., Kernbaum,S., Sicard,D., Buisson,Y., Agut,H., Escande,J.P., and Huraux,J.M. (1997). Exacerbations of clinical symptoms in human immunodeficiency virus type 1-infected patients with multicentric Castleman's disease are associated with a high increase in Kaposi's sarcoma herpesvirus DNA load in peripheral blood mononuclear cells. J. Infect. Dis. *175*, 1198-1201.

Greensill,J., Sheldon,J.A., Murthy,K.K., Bessonette,J.S., Beer,B.E., and Schulz,T.F. (2000a). A chimpanzee rhadinovirus sequence related to Kaposi's sarcoma-associated herpesvirus/human herpesvirus 8: increased detection after HIV-1 infection in the absence of disease. AIDS *14*, F129-F135.

Greensill,J., Sheldon,J.A., Renwick,N.M., Beer,B.E., Norley,S., Goudsmit,J., and Schulz,T.F. (2000b). Two distinct gamma-2 herpesviruses in African green monkeys: a second gamma-2 herpesvirus lineage among old world primates? J. Virol. *74*, 1572-1577.

Gruffat,H., Portes-Sentis,S., Sergeant,A., and Manet,E. (1999). Kaposi's sarcoma-associated herpesvirus (human herpesvirus-8) encodes a homologue of the Epstein-Barr virus bZip protein EB1. J. Gen. Virol. *80 (Pt 3)*, 557-561.

Hinnebusch,A.G. (1993). Gene-specific translational control of the yeast GCN4 gene by phosphorylation of eukaryotic initiation factor 2. Mol. Microbiol. *10*, 215-223.

Holbrook,J.D. and Danpure,C.J. (2002). Molecular basis for the dual mitochondrial and cytosolic localization of alanine:glyoxylate aminotransferase in amphibian liver cells. J Biol. Chem. *277*, 2336-2344.

Holloway,A.F., Occhiodoro,F., Mittler,G., Meisterernst,M., and Shannon,M.F. (2000). Functional interaction between the HIV transactivator Tat and the transcriptional coactivator PC4 in T cells. J. Biol. Chem. *275*, 21668-21677.

Honess,R.W. and Roizman,B. (1974). Regulation of herpesvirus macromolecular synthesis. I. Cascade regulation of the synthesis of three groups of viral proteins. J. Virol. *14*, 8-19.

Honess,R.W. and Roizman,B. (1975). Regulation of herpesvirus macromolecular synthesis: sequential transition of polypeptide synthesis requires functional viral polypeptides. Proc. Natl. Acad. Sci. U. S. A *72*, 1276-1280.

Hoppe-Seyler,F. and Butz,K. (1993). Repression of endogenous p53 transactivation function in HeLa cervical carcinoma cells by human papillomavirus type 16 E6, human mdm-2, and mutant p53. J. Virol. *67*, 3111-3117.

International Agency for Research on Cancer (1997). Epstein-Barr Virus and Kaposi's Sarcoma Herpesvirus/Human Herpesvirus 8. Lyon, France, 17-24 June 1997. IARC Monogr Eval. Carcinog. Risks Hum. *70*, 1-492.

Jang,S.K., Krausslich,H.G., Nicklin,M.J., Duke,G.M., Palmenberg,A.C., and Wimmer,E. (1988). A segment of the 5' nontranslated region of encephalomyocarditis virus RNA directs internal entry of ribosomes during in vitro translation. J Virol *62*, 2636-2643.

Jenner,R.G., Alba,M.M., Boshoff,C., and Kellam,P. (2001). Kaposi's sarcoma-associated herpesvirus latent and lytic gene expression as revealed by DNA arrays. J Virol *75*, 891-902.

Judde,J.G., Lacoste,V., Briere,J., Kassa-Kelembho,E., Clyti,E., Couppie,P., Buchrieser,C., Tulliez,M., Morvan,J., and Gessain,A. (2000). Monoclonality or oligoclonality of human herpesvirus 8 terminal repeat sequences in Kaposi's sarcoma and other diseases. J. Natl. Cancer Inst. *92*, 729-736.

Katano,H., Sato,Y., Kurata,T., Mori,S., and Sata,T. (2000). Expression and localization of human herpesvirus 8-encoded proteins in primary effusion lymphoma, Kaposi's sarcoma, and multicentric Castleman's disease. Virology *269*, 335-344.

Kliche,S., Nagel,W., Kremmer,E., Atzler,C., Ege,A., Knorr,T., Koszinowski,U., Kolanus,W., and Haas,J. (2001). Signaling by human herpesvirus 8 kaposin A through direct membrane recruitment of cytohesin-1. Mol. Cell *7*, 833-843.

Kohler,G. and Milstein,C. (1992). Continuous cultures of fused cells secreting antibody of predefined specificity. 1975. Biotechnology *24*, 524-526.

Kozak,M. (1991). Structural features in eukaryotic mRNAs that modulate the initiation of translation. J Biol. Chem. *266*, 19867-19870.

Kozak,M. (2002). Pushing the limits of the scanning mechanism for initiation of translation. Gene *299*, 1-34.

Kremmer,E., Kranz,B.R., Hille,A., Klein,K., Eulitz,M., Hoffmann-Fezer,G., Feiden,W., Herrmann,K., Delecluse,H.J., Delsol,G., Bornkamm,G.W., Mueller-Lantzsch,N., and Grassert,F.A. (1995). Rat monoclonal antibodies differentiating between the Epstein-Barr virus nuclear antigens 2A (EBNA2A) and 2B (EBNA2B). Virology *208*, 336-342.

Kwiatkowski,D.J., Mehl,R., and Yin,H.L. (1988). Genomic organization and biosynthesis of secreted and cytoplasmic forms of gelsolin. J Cell Biol. *106*, 375-384.

Lagunoff,M., Bechtel,J., Venetsanakos,E., Roy,A.M., Abbey,N., Herndier,B., McMahon,M., and Ganem,D. (2002). De novo infection and serial transmission of Kaposi's sarcoma-associated herpesvirus in cultured endothelial cells. J Virol *76*, 2440-2448.

Lagunoff,M. and Ganem,D. (1997). The structure and coding organization of the genomic termini of Kaposi's sarcoma-associated herpesvirus. Virology *236*, 147-154.

Li,H., Komatsu,T., Dezube,B.J., and Kaye,K.M. (2002). The Kaposi's sarcoma-associated herpesvirus K12 transcript from a primary effusion lymphoma contains complex repeat elements, is spliced, and initiates from a novel promoter. J. Virol. *76*, 11880-11888.

Lin,C.L., Li,H., Wang,Y., Zhu,F.X., Kudchodkar,S., and Yuan,Y. (2003). Kaposi's sarcoma-associated herpesvirus lytic origin (ori-Lyt)-dependent DNA replication: identification of the ori-Lyt and association of K8 bZip protein with the origin. J Virol *77*, 5578-5588.

Lin,K., Dai,C.Y., and Ricciardi,R.P. (1998). Cloning and functional analysis of Kaposi's sarcoma-associated herpesvirus DNA polymerase and its processivity factor. J. Virol. *72*, 6228-6232.

Lin,S.F., Robinson,D.R., Miller,G., and Kung,H.J. (1999). Kaposi's sarcoma-associated herpesvirus encodes a bZIP protein with homology to BZLF1 of Epstein-Barr virus. J. Virol. *73*, 1909-1917.

Low,W., Harries,M., Ye,H., Du,M.Q., Boshoff,C., and Collins,M. (2001). Internal ribosome entry site regulates translation of Kaposi's sarcoma-associated herpesvirus FLICE inhibitory protein. J Virol *75*, 2938-2945.

Lukac,D.M., Kirshner,J.R., and Ganem,D. (1999). Transcriptional activation by the product of open reading frame 50 of Kaposi's sarcoma-associated herpesvirus is required for lytic viral reactivation in B cells. J. Virol. *73*, 9348-9361.

Lukac,D.M., Renne,R., Kirshner,J.R., and Ganem,D. (1998). Reactivation of Kaposi's sarcoma-associated herpesvirus infection from latency by expression of the ORF 50 transactivator, a homolog of the EBV R protein. Virology *252*, 304-312.

Mayer,B.J. and Eck,M.J. (1995). SH3 domains. Minding your p's and q's. Curr. Biol. *5*, 364-367.

McGeoch,D.J. and Davison,A.J. (1999). The descent of human herpesvirus 8. Semin. Cancer Biol. *9*, 201-209.

Miller,G., Heston,L., Grogan,E., Gradoville,L., Rigsby,M., Sun,R., Shedd,D., Kushnaryov,V.M., Grossberg,S., and Chang,Y. (1997). Selective switch between latency and lytic replication of Kaposi's sarcoma herpesvirus and Epstein-Barr virus in dually infected body cavity lymphoma cells. J. Virol. *71*, 314-324.

Miller,G., Rigsby,M.O., Heston,L., Grogan,E., Sun,R., Metroka,C., Levy,J.A., Gao,S.J., Chang,Y., and Moore,P. (1996). Antibodies to butyrate-inducible antigens of Kaposi's sarcoma-associated herpesvirus in patients with HIV-1 infection. N. Engl. J. Med. *334*, 1292-1297.

Monini,P., Carlini,F., Sturzl,M., Rimessi,P., Superti,F., Franco,M., Melucci-Vigo,G., Cafaro,A., Goletti,D., Sgadari,C., Butto',S., Leone,P., Chiozzini,C., Barresi,C., Tinari,A., Bonaccorsi,A., Capobianchi,M.R., Giuliani,M., di Carlo,A., Andreoni,M., Rezza,G., and Ensoli,B. (1999). Alpha interferon inhibits human herpesvirus 8 (HHV-8) reactivation in primary effusion lymphoma cells and reduces HHV-8 load in cultured peripheral blood mononuclear cells. J. Virol. *73*, 4029-4041.

Moore,P. and Chang,Y. (2001). Kaposi's sarcoma associated virus. In Fields virology, D.M.Knipe, P.M.Howley, and a.et, eds. (Philadelphia, Pa.: Lippincott Williams & Wilkins), pp. 2803-2834.

Moses,A.V., Fish,K.N., Ruhl,R., Smith,P.P., Strussenberg,J.G., Zhu,L., Chandran,B., and Nelson,J.A. (1999). Long-term infection and transformation of dermal microvascular endothelial cells by human herpesvirus 8. J. Virol. *73*, 6892-6902.

Muralidhar,S., Pumfery,A.M., Hassani,M., Sadaie,M.R., Azumi,N., Kishishita,M., Brady,J.N., Doniger,J., Medveczky,P., and Rosenthal,L.J. (1998). Identification of kaposin (open reading frame K12) as a Human Herpesvirus 8 (Kaposi's Sarcoma-Associated Herpesvirus) transforming gene. J. Virol. *72*, 4980-4988.

Muralidhar,S., Veytsmann,G., Chandran,B., Ablashi,D., Doniger,J., and Rosenthal,L.J. (2000). Characterization of the human herpesvirus 8 (Kaposi's sarcoma-associated herpesvirus) oncogene, kaposin (ORF K12). J Clin. Virol *16*, 203-213.

Nagel,W., Zeitlmann,L., Schilcher,P., Geiger,C., Kolanus,J., and Kolanus,W. (1998). Phosphoinositide 3-OH kinase activates the beta2 integrin adhesion pathway and induces membrane recruitment of cytohesin-1. J. Biol. Chem. *273*, 14853-14861.

Nealon,K., Newcomb,W.W., Pray,T.R., Craik,C.S., Brown,J.C., and Kedes,D.H. (2001). Lytic replication of Kaposi's sarcoma-associated herpesvirus results in the formation of multiple capsid species: isolation and molecular characterization of A, B, and C capsids from a gammaherpesvirus. J. Virol. *75*, 2866-2878.

Neipel,F., Albrecht,J.C., and Fleckenstein,B. (1997). Cell-homologous genes in the Kaposi's sarcoma-associated rhadinovirus human herpesvirus 8: determinants of its pathogenicity? J. Virol. *71*, 4187-4192.

Oda,T., Funai,T., and Ichiyama,A. (1990). Generation from a single gene of two mRNAs that encode the mitochondrial and peroxisomal serine:pyruvate aminotransferase of rat liver. J Biol. Chem. *265*, 7513-7519.

Orenstein,J.M., Alkan,S., Blauvelt,A., Jeang,K.T., Weinstein,M.D., Ganem,D., and Herndier,B. (1997). Visualization of human herpesvirus type 8 in Kaposi's sarcoma by light and transmission electron microscopy. AIDS *11*, F35-F45.

Parravicini,C., Chandran,B., Corbellino,M., Berti,E., Paulli,M., Moore,P.S., and Chang,Y. (2000). Differential viral protein expression in Kaposi's sarcoma-associated herpesvirus-infected diseases: Kaposi's sarcoma, primary effusion lymphoma, and multicentric Castleman's disease. Am. J. Pathol. *156*, 743-749.

Paulose-Murphy,M., Ha,N.K., Xiang,C., Chen,Y., Gillim,L., Yarchoan,R., Meltzer,P., Bittner,M., Trent,J., and Zeichner,S. (2001). Transcription program of human herpesvirus 8 (kaposi's sarcoma-associated herpesvirus). J. Virol. *75*, 4843-4853.

Penn,I. (1978). Kaposi's sarcoma in organ transplant recipients: report of 20 cases. Transplantation *27*, 8-11.

Rabkin,C.S., Janz,S., Lash,A., Coleman,A.E., Musaba,E., Liotta,L., Biggar,R.J., and Zhuang,Z. (1997). Monoclonal origin of multicentric Kaposi's sarcoma lesions. N. Engl. J. Med. *336*, 988-993.

Rawlinson,W.D., Farrell,H.E., and Barrell,B.G. (1996). Analysis of the complete DNA sequence of murine cytomegalovirus. J. Virol. *70*, 8833-8849.

Regamey,N., Tamm,M., Wernli,M., Witschi,A., Thiel,G., Cathomas,G., and Erb,P. (1998). Transmission of human herpesvirus 8 infection from renal-transplant donors to recipients. N. Engl. J. Med. *339*, 1358-1363.

Ren,R., Mayer,B.J., Cicchetti,P., and Baltimore,D. (1993). Identification of a ten-amino acid proline-rich SH3 binding site. Science *259*, 1157-1161.

Renne,R., Lagunoff,M., Zhong,W., and Ganem,D. (1996a). The size and conformation of Kaposi's sarcoma-associated herpesvirus (human herpesvirus 8) DNA in infected cells and virions. J. Virol. *70*, 8151-8154.

Renne,R., Zhong,W., Herndier,B., McGrath,M., Abbey,N., Kedes,D., and Ganem,D. (1996b). Lytic growth of Kaposi's sarcoma-associated herpesvirus (human herpesvirus 8) in culture. Nat. Med. *2*, 342-346.

Reschke,M. (1994). Detailed 2d model of HCMV. Institute of Virology, University of Marburg, Germany.

Roizman,B. (1996). Herpesviridae. In Fields virology, B.N.Fields, D.M.Knipe, and P.M.Howley, eds. (Philadelphia, Pa.: Lippincott-Raven), pp. 2221-2230.

Russo,J.J., Bohenzky,R.A., Chien,M.C., Chen,J., Yan,M., Maddalena,D., Parry,J.P., Peruzzi,D., Edelman,I.S., Chang,Y., and Moore,P.S. (1996). Nucleotide sequence of the Kaposi sarcoma-associated herpesvirus (HHV8). Proc. Natl. Acad. Sci. U. S. A. *93*, 14862-14867.

Sadler,R., Wu,L., Forghani,B., Renne,R., Zhong,W., Herndier,B., and Ganem,D. (1999). A complex translational program generates multiple novel proteins from the latently expressed Kaposin (K12) locus of Kaposi's sarcoma-associated herpesvirus. J. Virol. *73*, 5722-5730.

Sarid,R., Flore,O., Bohenzky,R.A., Chang,Y., and Moore,P.S. (1998). Transcription mapping of the Kaposi's sarcoma-associated herpesvirus (human herpesvirus 8) genome in a body cavity-based lymphoma cell line (BC-1). J. Virol. *72*, 1005-1012.

Sarid,R., Wiezorek,J.S., Moore,P.S., and Chang,Y. (1999). Characterization and cell cycle regulation of the major Kaposi's sarcoma-associated herpesvirus (human herpesvirus 8) latent genes and their promoter. J Virol *73*, 1438-1446.

Schlessinger,J. (1994). SH2/SH3 signaling proteins. Curr. Opin. Genet. Dev. *4*, 25-30.

Seaman,W.T., Ye,D., Wang,R.X., Hale,E.E., Weisse,M., and Quinlivan,E.B. (1999). Gene expression from the ORF50/K8 region of Kaposi's sarcoma-associated herpesvirus. Virology *263*, 436-449.

Searles,R.P., Bergquam,E.P., Axthelm,M.K., and Wong,S.W. (1999). Sequence and genomic analysis of a Rhesus macaque rhadinovirus with similarity to Kaposi's sarcoma-associated herpesvirus/human herpesvirus 8. J. Virol. *73*, 3040-3053.

Siegal,B., Levington-Kriss,S., Schiffer,A., Sayar,J., Engelberg,I., Vonsover,A., Ramon,Y., and Rubenstein,E. (1990). Kaposi's sarcoma in immunosuppression. Possibly the result of a dual viral infection. Cancer *65*, 492-498.

Simas,J.P. and Efstathiou,S. (1998). Murine gammaherpesvirus 68: a model for the study of gammaherpesvirus pathogenesis. Trends Microbiol. *6*, 276-282.

Staskus,K.A., Zhong,W., Gebhard,K., Herndier,B., Wang,H., Renne,R., Beneke,J., Pudney,J., Anderson,D.J., Ganem,D., and Haase,A.T. (1997). Kaposi's sarcoma-associated herpesvirus gene expression in endothelial (spindle) tumor cells. J. Virol. *71*, 715-719.

Sturzl,M., Blasig,C., Schreier,A., Neipel,F., Hohenadl,C., Cornali,E., Ascherl,G., Esser,S., Brockmeyer,N.H., Ekman,M., Kaaya,E.E., Tschachler,E., and Biberfeld,P. (1997). Expression of HHV-8 latency-associated T0.7 RNA in spindle cells and endothelial cells of AIDS-associated, classical and African Kaposi's sarcoma. Int. J. Cancer *72*, 68-71.

Sun,R., Lin,S.F., Gradoville,L., Yuan,Y., Zhu,F., and Miller,G. (1998). A viral gene that activates lytic cycle expression of Kaposi's sarcoma-associated herpesvirus. Proc. Natl. Acad. Sci. U. S. A *95*, 10866-10871.

Sun,R., Lin,S.F., Staskus,K., Gradoville,L., Grogan,E., Haase,A., and Miller,G. (1999). Kinetics of Kaposi's sarcoma-associated herpesvirus gene expression. J. Virol. *73*, 2232-2242.

Tomkowicz,B., Singh,S.P., Cartas,M., and Srinivasan,A. (2002). Human herpesvirus-8 encoded Kaposin: subcellular localization using immunofluorescence and biochemical approaches. DNA Cell Biol. *21*, 151-162.

Virgin,H.W., Latreille,P., Wamsley,P., Hallsworth,K., Weck,K.E., Dal,C.A., and Speck,S.H. (1997). Complete sequence and genomic analysis of murine gammaherpesvirus 68. J. Virol. *71*, 5894-5904.

Wabinga,H.R., Parkin,D.M., Wabwire-Mangen,F., and Mugerwa,J.W. (1993). Cancer in Kampala, Uganda, in 1989-91: changes in incidence in the era of AIDS. Int. J. Cancer *54*, 26-36.

Wu,L., Lo,P., Yu,X., Stoops,J.K., Forghani,B., and Zhou,Z.H. (2000). Three-dimensional structure of the human herpesvirus 8 capsid. J. Virol. *74*, 9646-9654.

Zhong,W., Wang,H., Herndier,B., and Ganem,D. (1996). Restricted expression of Kaposi sarcoma-associated herpesvirus (human herpesvirus 8) genes in Kaposi sarcoma. Proc. Natl. Acad. Sci. U. S. A *93*, 6641-6646.

Zhu,F.X., Cusano,T., and Yuan,Y. (1999). Identification of the immediate-early transcripts of Kaposi's sarcoma-associated herpesvirus. J. Virol. *73*, 5556-5567.

7 Abbreviations

Å	Angström
A, Ade	adenine
AIDS	aquired immune deficiency syndrome
AP-1	activator protein 1
APS	ammonium persulfate
ATCC	American Type Culture Collection
ATP	adenosine triphosphate
BCBL-1	body cavity-based lymphoma cell line 1
BCIP	5-bromo-4-chloro-3-indolyl-1-phosphate
bp	base pair
°C	degree Celsius
C	cytosine
dUTPase	Deoxyuridine 5'-triphosphate nucleotidohydrolase
DMEM	Dulbecco's modified Eagle medium
DMF	N,N-dimethylformamide
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DO	dropout
DR	direct repeats
dNTP	deoxynucleoside triphosphate
DTT	dithiothreitol
E. coli	Escherichia coli
e.g.	exempli gratia (Lat. = for instance)
et al.	et alii (Lat. = and others)
EDTA	ethylenediamine tetraacetic acid
eGFP	enhanced green fluorescent protein
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmatic reticulum
EtOH	ethanol
FCS	fetal calf serum
f.l.	full length
Fig.	figure
FITC	fluorescein isothiocyanate
g	gram
g	gravitation constant
G	guanine
Grb2	growth factor receptor-bound protein 2
h	hour(s)
HAc	acetic acid
HCMV	human cytomegalovirus
HEPES	2-[4-(2-Hydoxyethyl)-1-piperazinyl]-ethane sulfonic acid
HHV-8	human herpesvirus-8
His	Histidine
HIV	human immunodeficiency virus
HSV	herpes simplex virus

IF	immunofluorescence
IFN	interferon
IgG	immunglobulin G
IL	interleukin
IP	immunoprecipitation
IPTG	isopropylthiogalactoside
ISRE	IFN-stimulated response element
kb	kilo bases
kbp	kilo base pairs
kd	kilodaltons
KS	Kaposi's Sarcoma
KSHV	Kaposi's Sarcoma associated herpesvirus
l	liter
LANA	Latency-Associated Nuclear Antigen
LB	Luria-Bertani
Leu	leucine
LTR	long terminal repeats
µ	micro (10^{-6})
m	milli (10^{-3})
m	meter
M	mol/liter, molar
MCMV	murine cytomegalovirus
mab	monoclonal antibody
MCD	multicentric Castleman's disease
MCP	major capsid protein
min	minute(s)
MOPS	3- (N-Morpholino)propanesulfonic acid
mRNA	messenger RNA
n	nano (10^{-9})
NB	NaCl/bicine
NBT	nitro blue tetrazolium
NFκB	nuclear factor kB
NLS	nuclear localization signal
o/n	overnight
OD	optical density
ORF	open reading frame
p	pico (10^{-12})
PAGE	polyacrylamide gel electrophoresis
PBS	phoshate buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
PEL	primari effusion lymphoma
PMSF	phenylmethylsulfonfluoride
prec.	precipitation
RNA	ribonucleic acid
rpm	revolutions per minute
RPMI	Rosswell Park Memorial Institute
RT	room temperature
s	second(s)
SBEG	sorbitol/bicine/ethylene glicol
SD	synthetic defined

SDS	sodium dodecylsulfate
SIV	simian immunodeficiency virus
SV-40	Simian virus 40
Tab.	Table
TAE	Tris-Acetate-EDTA
TBST	Tris buffered saline with Tween 20
TEMED	N, N, N', N'-tetramethylenediamine
T	thymine
TPA	12-O-tetradecanoylphorbol-13-acetate
Tris	Tris(hydroxymethyl)aminomethan
Trp	Tryptophane
U	unit(s), enzyme activity
untr	untransfected
UV	ultraviolet
V	Volt
VZV	varicella zoster virus
v/v	volumen/volumen
WB	Western blot
w/v	weight/volumen
wt	wild type
WW domain	domain that contains 2 conserved tryptophans and binds proline rich proteins
SH3 domain	Src homology 3 domain
X-α-Gal	5-Bromo-4-chloro-3-indolyl-α-D-galactopyranoside
YPD	yeast extract/peptone/dextrose

8 Curriculum Vitae

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Ausbildung:

1981-1990	Spohn-Gymnasium, Ravensburg
Mai 1990	Abitur
1990-1992	Wehrdienst in Stetten a.k.M., Sigmaringen und Aachen
1992-1999	Studium der Biochemie (Diplom) an der Eberhard-Karls-Universität Tübingen
Januar-April 1996	Studienaufenthalt am H.E.J. Research Institute of Chemistry der Universität Karachi, Pakistan: Aufreinigung und Charakterisierung von Naturstoffen
August 1998-April 1999	Diplomarbeit am Max-Planck-Institut für Biologie, Abt. Membranbiochemie, Tübingen, Prof. Dr. P. Overath Thema: Der Einfluß von Leishmania-Glykokonjugaten auf den Aktivierungszustand von Makrophagen
April 1999	Abschluß: Diplom
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Januar 2000-	Dissertation am Max-von-Pettenkofer-Institut, Genzentrum der Ludwig-Maximilians-Universität München unter der Betreuung von Prof. Dr. R. Grosschedl und der Anleitung von PD Dr. Dr. J. Haas. Thema: Translational initiation controls localization and regulatory function of the γ-herpesviral protein *kaposin*