Functional characterization of the M-type K15-encoded membrane protein of Kaposi’s sarcoma-associated herpesvirus

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Kaposi’s sarcoma-associated herpesvirus (KSHV) or human herpesvirus 8 is the causative agent of Kaposi’s sarcoma, primary effusion lymphoma and the plasma-cell variant of multicentric Castleman’s disease. Its alternatively spliced K15 gene encodes several membrane proteins with varying numbers of transmembrane domains. Two highly diverged alleles of the K15 gene, termed predominant (P) and minor (M), exist and share only 33% amino acid identity with one another, but retain conserved putative src homology (SH) 2- and SH3-binding motifs. K15-M is thought to have entered the KSHV genome as the result of recombination with a related \(\gamma_2\)-herpesvirus. The more common K15-P allele has been shown to activate the mitogen-activated protein kinases Erk2 and JNK1 and the nuclear factor \(\kappa B\) (NF-\(\kappa B\)) pathway. To explore possible functional differences between K15-P and K15-M that might have influenced their spread in the KSHV population, here, the ability of the M form of K15 to activate these pathways was investigated. Similarly to K15-P, K15-M induces the activation of the Erk2 and JNK1 kinases, the NF-\(\kappa B\) transcription factor and the expression of a similar range of cellular inflammatory genes, as assessed by gene-expression microarray studies and reporter assays. In epithelial cells, the activation of most K15-M target genes is impaired by mutagenesis of Y490 in its SH2-binding motif Y490EEV, although this motif appears less important in endothelial cells. Therefore, K15-M and K15-P can trigger similar intracellular signalling pathways, despite their extensive sequence divergence.

INTRODUCTION

Kaposi’s sarcoma-associated herpesvirus (KSHV) or human herpesvirus 8, discovered by Chang et al. (1994), is the only currently known human \(\gamma_2\)-herpesvirus (rhadinovirus). KSHV is associated with two lymphoproliferative diseases: primary effusion lymphoma (PEL) (Cesarman et al., 1995) and multicentric Castleman’s disease (MCD) (Soulier et al., 1995). KSHV is indispensable for the development of Kaposi’s sarcoma (KS), as shown by the detection of KSHV by PCR, immunohistology or in situ hybridization in virtually all KS tumours (Schulz, 1999). KS is, however, markedly more frequent in human immunodeficiency virus (HIV)-infected persons and in immunosuppressed transplant recipients (Schulz, 1999), suggesting that other factors, such as immune suppression or coinfection with HIV, dramatically accelerate the development of this tumour in a KSHV-infected individual (Schulz, 2006). Similar to other herpesviruses, KSHV exhibits both latent and lytic replication, both of which are characterized by distinct gene-expression programmes. Although KSHV characteristically remains latent in most infected cells, a small population of virus-infected cells can undergo productive (lytic) virus replication (Rainbow et al., 1997; Dupin et al., 1998; Sturzl et al., 1999; Katano et al., 2000; Parravicini et al., 2000).

A family of alternatively spliced transcripts originates from an eight-exon-containing gene, open reading frame (ORF) K15, located between ORF75 and the terminal repeats at the ‘right’ end of the viral genome in the convention adopted.
for the γ2-herpesvirus subfamily (Russo et al., 1996; Glenn et al., 1999; Poole et al., 1999; Choi et al., 2000). At least three highly diverged alleles of ORF K15, P, M and N, have so far been identified in different virus isolates and are thought to have arisen as the result of recombination events between distinct primate γ2-herpesviruses (Glenn et al., 1999; Poole et al., 1999; Alagiozoglou et al., 2000; Lacoste et al., 2000; Kakoola et al., 2001; Meng et al., 2001; Zong et al., 2002; Whitby et al., 2004; Hayward & Zong, 2007). The most frequently found P (predominant) form of K15, and the rarer M (minor) form, have been found worldwide (Poole et al., 1999; Lacoste et al., 2000; Kakoola et al., 2001; Meng et al., 2001). A third allele, N, has so far been only found in South Africa (Alagiozoglou et al., 2000; Zong et al., 2002; Whitby et al., 2004).

Despite their extensive sequence divergence, reflecting their presumed origin in different γ2-herpesviruses, the intron-exon structure and splicing patterns of the K15-P and K15-M alleles are very similar (Glenn et al., 1999). Among the multiple K15 mRNA splice variants documented so far for the K15-P or the K15-M allele, the longest, and apparently predominant, contains eight exons and is predicted to encode a membrane protein with 12 transmembrane segments and a C-terminal cytoplasmic domain (Glenn et al., 1999; Choi et al., 2000; Sharp et al., 2002; Brinkmann et al., 2003). The K15-P version of this protein shares only 33% amino acid identity with its K15-M counterpart (Glenn et al., 1999; Poole et al., 1999). In spite of the high sequence divergence between K15-P and K15-M, several sequence motifs, thought to represent src homology (SH) 2-, SH3- and putative tumour necrosis factor receptor-associated factor (TRAF)-binding sites in the cytoplasmic domains of the K15 proteins, are conserved, suggesting shared functional properties (Glenn et al., 1999; Choi et al., 2000; Brinkmann et al., 2003). The location of K15 in the viral genome, the predicted structure of the longest protein (12 transmembrane segments and a cytoplasmic domain) and the presence of putative SH2- and SH3-binding sites are reminiscent of features found in latent membrane protein 2A (LMP2A) of Epstein–Barr virus, which is thought to provide a survival signal for latently infected B cells and also to control the activation of the B-cell receptor and virus latency in B cells (Brinkmann & Schulz, 2006).

K15-derived proteins have been detected in B cells of MCD tumours and in latently infected PEL cell lines (Sharp et al., 2002). However, Northern blot, RT-PCR and gene-array studies on PEL cell lines indicate that the expression of K15 may be upregulated during the lytic cycle (Glenn et al., 1999; Choi et al., 2000; Jenner et al., 2001; Paulose-Murphy et al., 2001; Nakamura et al., 2003). We have recently shown the expression of a 45 kDa K15-P protein in an epithelial cell line stably transfected with a recombinant KSHV genome in a bacterial artificial chromosome vector, expression of which is upregulated upon induction of the lytic viral life cycle (Brinkmann et al., 2007). This 45 kDa K15-P protein is also seen upon transfection of an expression vector containing all eight K15-P exons and is therefore expected to contain 12 transmembrane domains and the cytoplasmic domain (Glenn et al., 1999; Choi et al., 2000; Brinkmann et al., 2003, 2007).

K15-P activates the mitogen-activated protein kinase (MAPK) pathway Ras/MEK/Erk2 and the c-Jun N-terminal kinase (JNK), as well as the transcription factor nuclear factor κB (NF-κB) (Brinkmann et al., 2003). Activation of these pathways involves phosphorylation of the SH2-binding motif Y481EEV by members of the src family of protein tyrosine kinases (PTKs) (Brinkmann et al., 2003). We have reported recently that downstream cellular targets of K15-P-induced signalling include a wide range of inflammatory cytokines, as well as downstream targets of vascular endothelial growth factor (VEGF) (Brinkmann et al., 2007). So far, no functional data on the M type of K15 have been reported.

In this study, therefore, we investigated the function of K15-M of KSHV, in particular its ability to initiate intracellular signal transduction. We have found that K15-M activates NF-κB, as well as the MAPKs Erk2 and JNK1, and that its downstream targets are similar to those activated by K15-P. However, a potential SH2-binding site, YEEV, which is conserved in K15-P (Y481EEV) and K15-M (Y490EEV), seems to be less critical for activation of the JNK pathway by K15-M compared with K15-P in epithelial cells, whilst in SLK endothelial cells, mutations of this motif in both K15-P and K15-M are compatible with the activation of several cellular promoters. Therefore, in spite of the substantial sequence divergence between K15-P and K15-M, their functional properties appear to be very similar.

**METHODS**

**Cells, transfections and inhibitors.** HEK (human embryonic kidney) 293-T, HeLa and human endothelial SLK cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 50 IU penicillin ml⁻¹ and 50 μg streptomycin ml⁻¹ at 37 °C in humidified air with 5% CO₂. 293-T and HeLa cells were transfected with FuGENE (Roche) and SLK cells with Lipofectamine 2000 (Invitrogen). The MEK1/2 inhibitors PD98059 and UO126 (Calbiochem), dissolved in DMSO, were used at a final concentration of 50 μM and added to the medium 14 h after transfection. Cells were incubated for another 8 h before lysis.

**DNA constructs.** Full-length K15-M cDNA (exons 1-8, aa 1-498) was generated from HBL-6 cells by reverse transcription with primer Lampcrev (5'-TACTTCTCCACACAGGCCC) and PCR amplification with forward primer LampnestforB (5'-TTGGCCCATGAAAT-TACAAAAATAC), binding to the putative start codon (underlined) of K15-M, and reverse primer Lampnestrev (5'-AGGTTAGCTCGTGGGAAACAAAC), binding to the putative stop codon (underlined) of K15-M. The resulting PCR product was cloned into pGEM-T (Promega). A C-terminal FLAG tag (underlined) was added to the K15-M cDNA by using primers LampnestforA (5'-GGGGATCCATGAAAT-TACAAAAATAC-3') and mflagLAMPNruI3 (5'-TATTCCCGATTCTGGTCATGTCCTTTGATCGTCCGGAGAACAAAAC-3'). The resulting PCR product was cloned into the eukaryotic expression vector pFJE4 and sequenced. The mutant K15-M Y490P was generated by PCR amplification of K15-M-FLAG with

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primers LampnestforA and flagK15MNruiY : F (5’-TATTCCGGCAT-TACCTTGCGATCTATCGTCTTTGTAGCTGCTGGGAAACAAACTTCTCCTAAACACCTC-3’; point mutation shown in bold). The PCR product was cloned into pJFJE and sequenced.

The C-terminally haemagglutinin epitope (HA)-tagged version of K15-M was generated by cloning the complete K15-M cDNA in the pVR1255-2xA vector using primers K15-MKozakNotIfor (5’-TA-TGCGCCGGCCGACCATGATACAAAAATACCTG-3’) and K15-MnoStopNotRev (5’-ATGGGCCCCGGCTCGCGGGAAACAAACTTCTC-3’). The K15-P expression vectors (Choi et al., 2000; Brinkmann et al., 2007) and expression vectors for pSRs-HA-JNK1 and pSRs-HA-Erk2 (Brinkmann et al., 2003) have been described previously. Glutathione S-transferase (GST)–c-Jun purification was also done as described previously (Brinkmann et al., 2003). The NF-κB reporter p3ENhBcoA-Luc, containing three NF-κB sites upstream of the luciferase gene, was expressed in 293-T cells (Immunotools). The cells were lysed in RIPA100 buffer to control for protein loading. Supernatants were analysed by ELISA according to the manufacturer’s instructions (Promega). Luciferase activity was measured in cleared lysates with a luciferase system in accordance with the manufacturer’s instructions (Promega). Luciferase activity was calculated as fold induction compared with mock (empty expression vector)-transfected controls.

**Oligonucleotide DNA microarray experiments.** HeLa cells were seeded in six-well plates at a density of 8 × 10⁴ cells per well and transfected 24 h later with 1 μg DNA per well. Thirty-two hours post-transfection, cells were lysed for RNA extraction according to the manufacturer’s instructions (Qiagen). For microarray experiments, a Human Inflammation Array (MWG Biotech) was used (distributed by Ocimum Biosolutions as Human Inflammation OciChip). A Human Inflammation Array contains 155 validated oligonucleotide probes for 136 inflammatory and 19 ‘housekeeping’ genes. Total RNA was purified with an RNeasy kit followed by ‘on-column’ DNase I digestion (Qiagen). RNA was used to prepare Cy3- or Cy5-labelled cRNA by oligo(dt)–T7-prime-stranded cDNA synthesis (Roche), followed by in vitro transcription with T7 polymerase (MegaScrypt T7 kit; Ambion) as directed by the manufacturers. cRNA yields were determined photometrically.

cRNAs were fragmented, repurified and hybridized to microarrays in pre-prepared hybridization solution (MWG Biotech) at 42 °C overnight and then washed sequentially in 2 × SSC, 1 % SDS, 1 × SSC and 0.5 × SSC. Hybridized arrays were scanned on an Affymetrix 428 scanner at variable PMT voltage settings. Fluorescence intensity values were processed by using Imagene 4.2 software (Biodiscovery). In order to obtain maximal signal intensities without saturation effects, intensity values from TIFF images were integrated into one value per probe by the MAVI software (version Pro 2.5.1; MWG Biotech). Data were filtered for flagged spots and low intensity values; remaining data were used to calculate ratios of gene expression by using Microsoft Excel macros. Additional information on the microarrays and methodology used can be obtained at http://www.mh-hannover.de/forschung/sfb566/microarray/index.phtml. The results shown in Fig. 5 are listed on this website with accession numbers G199 #2 and G217 #2.

**Cytokine ELISA.** HeLa cells were transfected as described for the DNA microarray. Thirty-two hours post-transfection, the conditioned medium was collected and centrifuged, and supernatants were analysed by ELISA according to the manufacturer’s instructions (Immunotools). The cells were lysed in RIPA100 buffer to control for K15 protein expression by immunoblotting.

**RESULTS**

**K15-M protein expression and subcellular localization.** Like its K15-P counterpart, the eight-exon-containing K15-M mRNA is predicted to encode a protein with 12 transmembrane segments and a C-terminal cytoplasmic domain featuring potential SH2- and SH3-binding motifs (Glenn et al., 1999; Poole et al., 1999), as illustrated schematically in Fig. 1(a). Expression vectors containing the K15-M cDNA (exons 1–8) with either an HA or a FLAG epitope tag fused to the C terminus were generated...
and expressed in HeLa cells. The K15-M protein was visualized by immunofluorescence with an antibody to the HA (Fig. 1b) or FLAG (not shown) epitope. K15-M showed a distinct, punctate expression pattern distributed evenly over the entire cell and localized to the perinuclear region (Fig. 1b), in a manner similar to transfected and endogenously expressed K15-P protein (Brinkmann et al., 2007). By immunoblotting of lysates from transiently transfected HeLa cells, the K15-M protein showed an apparent molecular mass of approximately 40 kDa (Fig. 1c), i.e. slightly smaller than the K15-P 45 kDa protein (Brinkmann et al., 2007). As we have observed previously for K15-P (Brinkmann et al., 2003, 2007), a more slowly migrating K15-M band of an apparent molecular mass consistent with a dimer was also seen in this immunoblot experiment (Fig. 1c), which avoided boiling the sample in SDS-PAGE loading buffer because of the propensity of K15-derived proteins to aggregate at higher temperatures (Brinkmann et al., 2003, 2007).

We therefore investigated the ability of K15-M to activate this pathway by virtue of an immunocomplex in vitro kinase assay. As depicted in Fig. 2(a), both K15-P and K15-M were able to induce the kinase activity of cotransfected HA-tagged Erk2 kinase in transiently transfected 293-T cells, as measured by phosphorylation of MBP.

The tyrosine residue 481 within the YEEV motif in K15-P is phosphorylated by src kinases and is required for the activation for the Erk2 and JNK1 pathways by K15-P in 293 cells (Choi et al., 2000; Brinkmann et al., 2003). This YEEV motif is conserved in K15-M (Glenn et al., 1999; Poole et al., 1999) (Fig. 1a). We therefore investigated its role in the activation of Erk2 by K15-M by mutating Y490 within the K15-M YEEV motif to F490, and analysed its ability to activate Erk2 as described above. The K15-M Y490F mutant is impaired, but not completely deficient, in its ability to activate Erk2 in the in vitro phosphorylation assay, whereas the K15-P Y481F mutant is completely inactive (Fig. 2a), as described previously (Brinkmann et al., 2003).

To demonstrate the specificity of the K15-M-induced phosphorylation of MBP by Erk2, we applied PD98059 and UO126, the chemical inhibitors of MEK1/2, the upstream kinase activating Erk1/2, in an immunocomplex kinase

**Activation of Erk2 and JNK1 by K15-M**

We have shown previously that K15-P can induce the kinase activity of the MAPK Erk2 (Brinkmann et al., 2003).
We found that addition of these inhibitors abolished the K15-M-induced activation of Erk2 (Fig. 2b). Therefore K15-M, like K15-P, activates Erk2 via an involvement of its SH2-binding site, Y490EEV.

By using a similar kinase assay, with GST-c-Jun protein as substrate for the MAPK JNK1, we could show that K15-M induced JNK1 activity strongly in cotransfected 293-T cells (Fig. 3, right panel). In contrast to the K15-P Y481F mutant, which was impaired in its ability to activate JNK1 (Fig. 3, left panel) (Brinkmann et al., 2003), the K15-M Y490F mutant was affected only moderately in comparison with wild-type K15-M (Fig. 3, right panel).

**Activation of cellular gene expression by K15-M**

As K15-P activates the NF-κB pathway (Brinkmann et al., 2003), we analysed the ability of K15-M to activate NF-κB by cotransfecting 293-T cells with the corresponding K15 expression vectors and a luciferase reporter plasmid containing three NF-κB-responsive sites. As shown in Fig. 4(a), both K15-P and K15-M showed a dose-dependent, up to 20-fold activation of the NF-κB reporter, whereas K15-P Y481F and K15-M Y490F were impaired significantly in their ability to activate NF-κB. This suggests an involvement of the conserved SH2-binding motif, probably via its phosphorylation by members of the PTK family, as shown previously for K15-P (Choi et al., 2000; Brinkmann et al., 2003), in the initiation of NF-κB signalling by K15-M.

K15-P and K15-M both activated a cyclin E reporter plasmid (Fig. 4b). Here, again, phosphorylation of the YEEV motif appears to be critical for both K15-P and K15-M to achieve substantial activation (Fig. 4b).

Using high-density gene-array experiments, we have found recently that K15-P induces a broad range of cellular genes activated during inflammation, as well as VEGF-activated signalling cascades (Brinkmann et al., 2007). We therefore used a gene array containing 136 inflammatory and 19 housekeeping genes to analyse the transcriptome patterns induced by K15-M in transiently transfected HeLa cells. Fig. 5 shows the averaged results of two experiments, revealing that K15-M induced a broad range of inflammatory cytokines, in particular interleukin-8 (IL-8), IL-6,
IL-1α, chemokine ligand 20 (ccl20) and chemokine ligand 3 (cxcl3), and the VEGF downstream targets Down syndrome critical region 1 (dscr1) and cyclooxygenase 2 (cox2/ptgs2). Superoxide dismutase 2 (sod2) and matrix metalloproteinase (mmp) 1 and 3 appear to be induced more strongly by K15-M than by K15-P (Fig. 5, upper panel). K15-M and K15-P protein expression in transfected HeLa cells was controlled by immunoblotting analysis (data not shown).

To confirm the results obtained with the inflammation array, ELISA assays for secreted cytokines and reporter assays for several cellular promoters were carried out. Tissue-culture supernatants from HeLa cells transfected with expression vectors for wild-type K15-M or K15-M Y490F were tested for IL-6 and IL-8 secretion by ELISA. As shown in Fig. 6(a), the secretion of both IL-6 and IL-8 was induced strongly by K15-M, but not by the K15-M Y490F mutant. Expression levels of K15-M and the K15-M Y490F mutant were controlled by immunoblotting (Fig. 6b).

The activation of the dscr1 and cox2 promoters by K15-M in HeLa cells was verified in luciferase reporter assays and found to be similar to the previously reported activation by K15-P (Fig. 7a) (Brinkmann et al., 2007). In HeLa cells, the activation of these cellular promoters by K15-M was impaired when the SH2-binding motif Y490EEV was mutated (Fig. 7a), again similar to what we observed for K15-P (Brinkmann et al., 2007) and as shown in Fig. 7(a) when tested in parallel to K15-M.

VEGF, an angiogenic cytokine that is thought to play an important role in the pathogenesis of KS (Biberfeld et al., 1998), can induce dscr1 expression via activation of the NFAT transcription factor (Hesser et al., 2004). We have recently found that K15-P can activate several downstream targets of VEGF without marked induction of VEGF expression itself and that it can induce the NFAT transcription factor (Brinkmann et al., 2007). We therefore investigated whether K15-M would act in a similar manner. HeLa cells were transiently cotransfected with K15-P, K15-M and the corresponding YEEV mutants with the control luciferase reporter plasmid pTA, containing the TA promoter, or the reporter plasmid pNFAT-TA, containing NFAT-binding sites upstream of the TA promoter. Both types of K15 were found to upregulate this promoter construct significantly, whereas the corresponding YEEV mutants were impaired (Fig. 7b).

The K15-P Y481EEV motif is essential for the activation of most K15-P-induced signalling pathways and cellular genes in epithelial cells, although a few cellular genes are activated equally well by wild-type K15-P and the K15-P Y481F mutant (Brinkmann et al., 2003, 2007). However, in the endothelial SLK cell line, expression of dscr1 and cox2 and activation of NFAT, which are dependent on the intact K15-P Y481EEV motif in epithelial cells, are activated equally well by wild-type K15-P and the Y481F mutant (Brinkmann et al., 2007). As shown in Fig. 7(c, d), K15-M appears to follow this pattern, as the K15-M Y490F mutant stimulates the dscr1 and cox2 promoters and NFAT activity to the same extent as wild-type K15-M in SLK cells, whilst a clear difference between these two constructs can be seen in HeLa cells (Fig. 7a, b).

**DISCUSSION**

The fact that three different alleles of K15, i.e. K15-P, K15-M and K15-N, should occur in different KSHV isolates, presumably as a result of recombination events between KSHV and an as-yet-unknown γ2-herpesvirus (Glenn et al., 1999; Poole et al., 1999; Lacoste et al., 2000; Kakoola et al., 2001), raises the question as to whether they differ with regard to functional properties and, if such differences exist, whether they could have contributed to the spread of one or the other allele in the KSHV population. Of these...
three K15 alleles, K15-P is found most frequently and is therefore thought to represent the 'original' KSHV allele (Poole et al., 1999). However, other scenarios, such as K15-P having spread more efficiently due to a (hypothetical) increased fitness and having therefore replaced a prototypic K15-M, cannot currently be excluded. Like K15-P, K15-M has also been found in KSHV isolates of different geographical regions, and two different K15-M lineages, K15-M and K15-M', have been identified in Africa (Lacoste et al., 2000; Kakoola et al., 2001), suggesting that it recombined into KSHV several thousand years ago and has since undergone further evolution. In contrast, K15-N has so far only been observed in rare samples from South Africa (Alagiozoglou et al., 2000; Zong et al., 2002). K15-P and K15-M are also more divergent than K15-P and K15-N (M. Schmidt, M. Dedicoat & T. F. Schulz, unpublished data) and share only 33 % protein sequence identity (Glenn et al., 1999; Poole et al., 1999; Kakoola et al., 2001). Sequence motifs that could represent SH2- and SH3-binding sites are, however, conserved in K15-P and K15-M (Fig. 1a).

So far, functional properties have only been investigated for K15-P (Glenn et al., 1999; Choi et al., 2000; Sharp et al., 2002; Brinkmann et al., 2003, 2007). K15-P has been shown to activate several cellular signalling pathways and to induce a range of cellular genes frequently upregulated during inflammation, suggesting that it may contribute to the increased expression of inflammatory cytokines often found in KS lesions (Brinkmann et al., 2003, 2007). K15-P also interacts with a range of cellular proteins involved in signal transduction, including TRAFs 1, 2 and 3, several members of the src family of PTKs and HAX-1, a protein implicated in the regulation of apoptosis (Glenn et al., 1999; Choi et al., 2000; Sharp et al., 2002; Brinkmann et al., 2003). In this study, we explored whether K15-P and K15-M differ with respect to their ability to activate cellular signalling cascades and cellular gene expression.

To carry out such functional studies, K15-M was expressed in a range of cell lines by transient transfection. Although the predicted protein size for a K15-M protein containing

![Fig. 5. K15-M and K15-P induce a similar range of inflammation-related cellular genes. HeLa cells were transfected with an expression vector for K15-M or K15-P or empty expression vector. Thirty hours after transfection, RNA was extracted and transcribed into cRNA, which was then hybridized to a gene-expression array containing 136 inflammation-related cellular genes. Relative activation of cellular genes by K15-M/K15-P in comparison to the empty expression vector is shown (K15-M/control, black bars; K15-P/control, grey bars). Relative activation values represent means±SEM from two independent experiments. Shown are the values for 25 genes, which were regulated by at least twofold in at least one of the replicate experiments (top panel). Asterisks indicate three genes that were expressed more strongly in K15-M-transfected cells. Bottom panel: no significant effect of K15-M or K15-P on the expression of 19 'housekeeping' genes was seen.](image-url)
all eight exons is 11 aa longer than that of the corresponding K15-P protein, we found that its apparent molecular mass of approximately 40 kDa on SDS-PAGE was smaller than that observed routinely for K15-P (45 kDa) (Brinkmann et al., 2003, 2007). The same apparent molecular mass of approximately 40 kDa for K15-M was obtained by using an expression vector containing an N-terminal tag (data not shown), thus excluding the

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**Fig. 6.** Induction of IL-6 and IL-8 secretion by K15-M. HeLa cells were transfected with expression constructs for K15-M, K15-M Y490F or empty vector, and tissue-culture supernatants were harvested 32 h after transfection. The concentration of IL-6 and IL-8 was measured by ELISA (a). After removal of the conditioned supernatant for the ELISA assay, HeLa cells were lysed and protein expression of K15-M and K15-M Y490F was analysed by immunoblotting with an antibody to the FLAG epitope tag (b).

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**Fig. 7.** Activation of cellular promoters in HeLa and SLK cells by K15-M. (a) Activation of the dscr1 and cox2 promoters by K15-M in epithelial HeLa cells requires an intact YEEV motif. HeLa cells were cotransfected with K15-M, K15-M Y490F, K15-P, K15-P Y481F or empty expression vector and luciferase reporter constructs containing the dscr1 or cox2 promoter elements or a promoterless reporter (pGL3b). (b) Activation of an NFAT-responsive promoter by K15-M in HeLa cells requires an intact YEEV motif. HeLa cells were cotransfected with K15-M, K15-M Y490F, K15-P, K15-P Y481F or empty expression vector and a luciferase reporter construct containing an NFAT-responsive element upstream of the TA promoter, or the control reporter containing the TA promoter only. (c) Activation of dscr1 and cox2 promoters by K15-M in SLK endothelial cells does not depend on an intact YEEV motif. SLK cells were cotransfected with K15-M, K15-M Y490F or empty expression vector and the dscr1, cox2 or pGL3b reporters. (d) Activation of NFAT by K15-M in SLK cells does not depend on an intact YEEV motif. SLK cells were cotransfected with K15-M, K15-M Y490F or empty expression vector and the NFAT–TA or TA reporters. Luciferase reporter assays in (a–d) were carried out as described in Methods. Relative activation compared with that in mock-transfected cells from duplicate samples from three independent experiments is shown.
possibility that the 40 kDa protein could be the result of inadvertent internal splicing of the mRNA generated with the transfected expression construct or of an internal translation initiation. As the apparent molecular mass of both the K15-P and K15-M proteins is smaller than predicted for proteins of 489 and 490 aa, respectively, aberrant migration on SDS-PAGE remains a possibility. We have shown recently that 293 cells harbouring an entire KSHV genome in a bacterial artificial chromosome vector produce a 45 kDa K15-P protein identical in apparent molecular mass to that obtained by transient transfection of a transfected expression vector similar to the one generated for K15-M in this study (Brinkmann et al., 2007). It is therefore very likely that the K15-M protein studied here for its functional properties is identical to the one produced in virus-infected cells.

As far as can be deduced from the in vitro assays employed in this study, these K15-M and K15-P proteins appear to be largely functionally equivalent. Both activate the MEK/Erk, JNK and NF-κB pathways, as measured by in vitro kinase and reporter assays. They also induce a similar range of cellular genes often implicated in inflammation, as measured by an inflammation-gene array, a cytokine ELISA and reporter assays. Minor differences between K15-P and K15-M may, however, exist with respect to how these signalling pathways are initiated. Whilst mutation of tyrosine 481 in the conserved YEEV motif abolishes activation of the MEK/Erk and JNK cascade by K15-P in 293-T cells and the expression of a range of cellular genes in HeLa cells (Brinkmann et al., 2003, 2007), the equivalent mutation of tyrosine 490 in K15-M is associated with only a moderate decrease in activation of the JNK pathway in epithelial cells. As shown previously for K15-P (Brinkmann et al., 2007), we noted in this study a difference between an epithelial and an endothelial cell line with respect to the requirement of the YEEV motif for the activation of the VEGF downstream targets dscr1, cox2 and NFAT. This difference suggests that details of the recruitment of upstream signalling mediators, such as src kinases, may differ depending on the cell lineage, although this difference has so far only been noted in a few cell lines, and a 'cell-line'-specific difference cannot be ruled out. Therefore, qualitatively at least, K15-M and K15-P appear to share similar functions in spite of their extensive sequence difference. So far, there appears to be no obvious K15-related function that would have favoured the spread of one of these two K15 alleles after K15-M entered a K15-P-containing KSHV population, presumably as the result of a recombination event with a related γ2-herpesvirus.

However, we cannot rigorously exclude the possibility that some of the minor quantitative differences observed between K15-P and K15-M (e.g. extent of promoter activation and functional importance of Y481 in K15-P versus Y490 in K15-M) in the cell culture-based assays employed here could have sufficed to favour the spread of one of these two alleles in the KSHV population.

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