Kaposi’s sarcoma-associated herpesvirus Lana-1 is a major activator of the serum response element and mitogen-activated protein kinase pathways via interactions with the Mediator complex

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In cells infected with Kaposi’s sarcoma-associated herpesvirus (KSHV), the activation of mitogen-activated protein kinase (MAPK) pathways plays a crucial role early after virus infection as well as during reactivation. In order to systematically identify viral proteins activating MAPK pathways in KSHV-infected cells, a clone collection of KSHV open reading frames (ORFs) was screened for induction of the serum response element (SRE), as SRE is induced by MAPKs. The strongest induction of the SRE was found with ORF73 (latency-associated nuclear antigen 1, or Lana-1), although weaker activation was also found with the kaposin B isoform, ORF54 (dUTPase) and ORF74 (G-protein-coupled receptor). The bipartite SRE is bound by a ternary complex consisting of serum response factor (SRF) and ternary complex factor. Lana-1 bound directly to SRF, but also to the MED25 (ARC92/ACID-1), MED15 (PCQAP) and MED23 (Sur-2) subunits of the Mediator complex, a multi-subunit transcriptional co-activator complex for RNA polymerase II. Lana-1-induced SRE activation was inhibited by the dominant-negative N-terminal domain of the MED25 mediator subunit, suggesting that this subunit mediates Lana-1-induced SRE activation. In summary, these data suggest a model in which Lana-1 acts as an adaptor between the transcription factor SRF and the basal transcriptional machinery.

## INTRODUCTION

Kaposi’s sarcoma-associated herpesvirus (KSHV; human herpesvirus 8) in the genus *Rhadinovirus*, subfamily *Gammaherpesvirinae*, is the causative agent of the angio-proliferative skin tumour Kaposi’s sarcoma and two B-cell lymphomas – primary effusion lymphoma and multicentric Castleman’s disease (Cesarman et al., 1995; Chang et al., 1994; Soulier et al., 1995). Kaposi’s sarcoma lesions are composed predominantly of spindle cells, which have a gene expression profile similar to that of lymphatic endothelial cells (LECs) (Hong et al., 2004; Wang et al., 2004). KSHV infection of LECs and blood vascular endothelial cells (BVECs) induces a transcriptional reprogramming of both cell types, thought to lead to the development of spindle cells and Kaposi’s sarcoma lesions (Hong et al., 2004; Wang et al., 2004).

The vast majority of spindle cells are latently infected and express only a small subgroup of viral proteins, including latency-associated nuclear antigen 1 (Lana-1), v-cyclin, v-FLIP and kaposin A (Dupin et al., 1999; Katano et al., 2000). Most other viral proteins are expressed only in lytically infected cells, which constitute <1% of the tumour. Of the latently expressed KSHV proteins, Lana-1, the protein product of open reading frame (ORF) 73, is expressed in all KSHV-infected cells investigated thus far (Kellam et al., 1997; Rainbow et al., 1997). As well as being essential for replication and maintenance of viral episomes, nuclear-localized Lana-1 binds to a variety of transcriptional regulators including RING3, ATF/CREB2 and CBP, and acts as a transcriptional activator or suppressor of cellular and viral genes via the acidic internal repeat domain (Barbera et al., 2006; Lim et al., 2000, 2001; Platt et al., 1999; Renne et al., 2001). To date, the function of the majority of KSHV proteins either is unknown or is only predicted due to sequence homology with other herpesvirus proteins.
virus proteins. Although many viral genes show homologies to cellular genes and share structural similarities, they possess distinct functions compared with their cellular counterparts. The precise mechanism by which KSHV induces tumour formation thus remains unclear.

The serum response element (SRE) is the dominant cis-element responding to KSHV infection, with one-third of genes upregulated in KSHV-infected BVECs found to contain an SRE in their promoter. The SRE is present in promoters of a variety of cellular genes involved in cell growth, differentiation, neuronal transmission and muscle development, and is activated by agents such as serum, cytokines, tumour necrosis factor alpha (TNF-α), hepatitis B virus activator protein pX, proto-oncogenes and extracellular stimuli (Chai & Tarnawski, 2002). The SRE is a bipartite binding site for a complex of the transcription factors serum response factor (SRF) and ternary complex factor (TCF), a family of Ets domain proteins that bind to sequence-specific Ets-binding sites (EBSs) adjacent to the SRE (Shaw & Saxton, 2003). The SRE is activated by two pathways. The TCF-dependent, or Ras-dependent, pathway involves the Ras–Raf–MEK–ERK signalling cascade, whereas the TCF-independent pathway (the Rho-dependent pathway) involves the Rho-family GTPases (reviewed by Posern & Treisman, 2006). TCFs are targets for mitogen-activated protein kinases (MAPKs) and thus link growth factor–activated signalling pathways with transcriptional regulation in eukaryotic cells (Shaw & Saxton, 2003).

The activation of MAPK pathways is crucial at several stages during KSHV infection. Their activation immediately following infection enables successful establishment of KSHV infection (Pan et al., 2006; Sharma-Walia et al., 2005) and, subsequently, MAPK pathways are activated during reactivation of latent infections (Ford et al., 2006; Xie et al., 2008; Yu et al., 2007). In order to identify KSHV proteins responsible for MAPK pathway activation, we performed a genome-wide screen with individual KSHV ORFs for proteins able to induce a luciferase reporter containing SREs. Here, we identified Lana-1 as a major activator of promoters containing SREs and showed that this activation is exerted through its binding to the Mediator complex (a multi-subunit transcriptional co-activator complex for RNA polymerase II) and the transcription factor SRF.

**RESULTS**

**Lana-1 is the major SRE activator in KSHV**

We recently generated a clone collection of KSHV ORFs by recombinatorial cloning (Uetz et al., 2006). The KSHV ORFeome was subcloned from the pDONR entry vector into the eukaryotic expression vector pCR3 to generate a viral protein library that can be expressed in mammalian cells. This KSHV expression library was used to screen for viral genes activating SRE, as SREs are activated by MAPKs and these kinase pathways are crucial following virus infection as well as for reactivation from latency. Eighty of the 89 individual viral ORFs were co-transfected into human embryonic kidney (HEK293) cells together with a luciferase reporter plasmid containing five copies of the SRE element derived from the promoter of the c-fos proto-oncogene (Lazo et al., 1992; Treisman, 1986). From this KSHV genome-wide screen, we found that ORF54 (dUTPase), ORF74 [G-protein coupled receptor (GPCR)] and kaposin B (isoform of a virulence/latency factor) activated SRE, showing a 10-fold, 8-fold and 12.5-fold increase above background levels, respectively, in comparison with the 35-fold increase observed with a known activator of SRE, Ras V12 (Fig. 1a). However, ORF73 (Lana-1) activated SRE approximately 20-fold compared with background levels and was thus found to be a considerably stronger inducer of SRE (Fig. 1a). Several other viral proteins induced a low-level SRE activation (2–4-fold) that was below the threshold. Both ORF74 and kaposin B are known to activate MAPKs (McCormick & Ganem, 2005; Smit et al., 2002). When increasing amounts of Lana-1 DNA (50–250 ng per well) were transfected into HEK293 cells, we detected a dose-dependent activation of SRE (Fig. 1b), suggesting that the activation by Lana-1 is specific. In addition to HEK293 cells, we determined whether Lana-1 activated SRE in a more physiologically relevant cell type such as endothelial cells, one of the natural target cells for KSHV in vivo. In contrast to the observations in HEK293 cells, where activation of SRE by Lana-1 was strong but consistently lower than that by Ras V12, we found that, in endothelial cells, Lana-1 activated SRE to levels similar to Ras V12 (~10-fold activation in comparison with ~13-fold activation by Ras V12) (Fig. 1c).

**Lana-1 is not a general processivity factor**

Next, we investigated the capacity of Lana-1 to activate a variety of other cellular and viral promoters, including AP-1 and NF-κB. Of the promoters tested here, only those containing SREs were activated by Lana-1 (Fig. 2a). To demonstrate that SRE stimulation by Lana-1 was specific and not due to general processivity factor activity, we tested the capacity of Lana-1 to further stimulate promoters pre-activated by physiological stimuli. Lana-1 could further increase the activity of SRE promoters pre-activated with Ras V12, but had no effect on the activity of pre-stimulated promoters containing other binding sites (Fig. 2b). This indicated that Lana-1 acts only on specific promoters and that it probably has a different mode of action to the transcription factors themselves (Fig. 2b).

**Lana-1 acts through both SRF- and TCF-binding sites**

The SRE element contains two distinct binding sites for SRF and TCF, which is a family of related transcription factors forming a ternary complex with two SRF molecules.
To evaluate the role of SRF and TCF in Lana-1-mediated induction of SRE, we tested c-fos promoter constructs with mutated SRF (mSRF) or TCF (mTCF) binding sites (Fig. 3a). Each plasmid contained a single copy of the wild-type or mutated SRE sequence. Ras V12-induced SRE activation can be mediated by TCF-dependent or independent pathways, both of which require SRF. As expected, dominant-positive Ras V12 was still able to activate the promoter with the mTCF-binding site, but not the promoter with the mSRF-binding site (Fig. 3b). Lana-1, however, was still able to activate both mutant promoters to approximately 50% of the level of the wild-type SRE promoter (Fig. 3b), suggesting that Lana-1 modulates both pathways leading to the activation of TCF and SRF transcription factors. By co-immunoprecipitation (CoIP) with anti-SRF antibodies, we were able to detect a direct interaction between Lana-1 and endogenous SRF (Fig. 4a). The same interaction, however, was not observed by CoIP with the anti-Lana-1 antibody. It is common in CoIP experiments for an interaction to occur in a single direction only, and in this instance was probably due to steric interference of the anti-Lana-1 antibody preventing binding of the SRF. In order to determine whether Lana-1 interacted specifically with SRF, we investigated its capacity to interact with other transcription factors, including the ternary complex factor ELK-1, and STAT-1 and Sp13. Following co-transfection of HA-tagged Lana-1 and Myc-tagged transcription factors, we were unable to detect an interaction of Lana-1 with the other transcription factors tested here by CoIP (Fig. 4b).

**Lana-1 activation of SRE requires MAPK pathways**

Ras-induced activation of SRE is mediated by a sequential phosphorylation of the Raf–MEK–ERK signalling pathway, and the ternary complex factor ELK-1 (Fig. 5a). We thus asked whether Lana-1 activation of SRE also involved this pathway. In luciferase assays with SRE reporter plasmids, we found that the two MEK1/2 inhibitors U0126 and PD98059 inhibited Lana-1-induced activation to a similar extent to Ras-induced activation (Fig. 5b).

**Lana-1 acts through the Mediator complex**

Transcriptional activation via the MAPK pathway requires binding of the ternary complex factor ELK-1 to the Sur-2 (suppressor of ras; MED23) subunit of the Mediator complex (Bourbon et al., 2004; Stevens et al., 2002). We thus hypothesized that the Mediator complex may also be involved in Lana-1-induced transcriptional activation. In fact, in CoIP experiments, Lana-1 bound to several endogenously expressed Mediator complex subunits including MED15 (PCQAP) and MED23 (Fig. 6a–c) (Bourbon et al., 2004). Lana-1 also interacted with both endogenous (Fig. 6b, lanes 1–4) and overexpressed (Fig. 6b,
lanes 5–8) MED25 (ARC92/ACID-1; human activator-recruited co-factor). To test whether binding to MED25 is crucial for Lana-1-mediated transactivation similar to the herpes simplex virus type 1 (HSV-1) transactivator VP16 (Bourbon et al., 2004; Mittler et al., 2003), we used the dominant-negative N-terminal domain (NTD) of MED25 in SRE–luciferase assays (Mittler et al., 2003). As reported previously, the NTD inhibited activation of a luciferase reporter plasmid containing Gal4-binding sites (pGLMRG5) by either full-length VP16 fused to Gal4 or the H1 domain of VP16 fused to Gal4 (Fig. 6d) (Mittler et al., 2003). In contrast, the NTD had no effect on the activation of SRE by Ras V12 (Fig. 6d), indicating that MED25 is not involved as a mediator complex subunit. However, Lana-1-induced SRE activation was strongly inhibited by the NTD (up to 84%) in a manner similar to that observed with the VP16 control, suggesting that MED25 binding is mandatory for transactivation.

The N terminus of Lana-1 binds to the Mediator complex subunit MED25

To identify the binding region of Lana-1 to the Mediator complex, we used histidine- and glutathione S-transferase (GST)-tagged proteins of Lana-1 in CoIPs and GST pull-down assays (Fig. 7a). Two C-terminal deletion mutants, L25 and L26, were still able to bind to MED25 by CoIP (Fig. 7a). However, the deletion of the C terminus removed the capacity of Lana-1 to activate the SRE (Fig. 7d), in accordance with previous observations that the C-terminal 34 aa of Lana-1 are required for activation of the Epstein–Barr virus (EBV) Cp promoter (Viejo-Borbolla et al., 2003). In addition to the C-terminal mutants above, Lana-1 mutants pGL9 and pGL13, containing both N and C termini or the N terminus alone, respectively, were able to bind MED25 in GST pull-down assays (Fig. 7c). However, the Lana-1 mutant pGL12, which lacked the N terminus and central domain, did not pull down MED25. These data strongly suggest that the...
N-terminal domain of Lana-1 is responsible for binding to MED25 of the Mediator complex, whilst the C terminus enables activation of the SRE. Taken together, these data lead to a model in which Lana-1 acts as an adaptor between specific transcription factors and the Mediator complex to modulate host-cell transcription in a manner similar to that recently reported for the varicella-zoster virus (VZV) transactivator IE62 (Fig. 8) (Yang et al., 2008).

DISCUSSION

During in vitro infection, KSHV induces a transcriptional reprogramming of BVECs and LECs, which results in a gene expression profile similar to that observed naturally in Kaposi’s sarcoma tumours (Hong et al., 2004; Wang et al., 2004). Promoters containing an SRE are found at high frequency in genes upregulated upon KSHV infection. As SREs are found in genes that regulate cell growth and differentiation, and can be induced by MAPKs, aberrant stimulation of this element could potentially play a role in these observed phenotypic alterations, as well as a general role in KSHV infection, reactivation and tumorigenesis. In this study, we functionally screened a genome-sized KSHV clone collection for viral proteins activating MAPKs and the SRE. Using a luciferase–SRE reporter assay, we confirmed two known MAPK activators, kaposin B and ORF74, and identified two previously unknown KSHV proteins as MAPK activators, ORF54 and Lana-1.

Both ORF74 and kaposin B, as well as the previously unidentified MAPK activator ORF54, were identified as low-level inducers of SRE, increasing activity up to 13-fold.
above background levels. ORF54 is a nuclear-localized functional dUTPase expressed during lytic replication (Kremmer et al., 1999). It is possible that ORF54 activates the SRE indirectly by upregulating synthesis of the mitogen TNF-α in a manner similar to EBV, another human gammaherpesvirus, whose dUTPase has been shown to upregulate production of several pro-inflammatory cytokines, including TNF-α (Glaser et al., 2006). ORF74 is a GPCR that constitutively activates p44/p42 MAPK and Akt, as well as the phosphoinositol 3-kinase (PI3K) pathway (Arvanitakis et al., 1997; McCormick & Ganem, 2005; Smit et al., 2002). The PI3K pathway and these MAPKs ultimately activate the Ras–Raf–MEK pathway, which may result in or contribute to the observed SRE activation by the viral GPCR. In addition, activation of the SRE, in combination with activation of the pro-inflammatory transcription factors AP-1 and NF-κB, may contribute to GPCR-mediated tumorigenesis (Montaner et al., 2003; Schwarz & Murphy, 2001; Smit et al., 2002).

In this screen, Lana-1 was identified as the major KSHV protein responsible for SRE activation, inducing up to 20-fold higher activity than background levels in a specific and dose-dependent manner (Figs 1 and 2). Even the presence of only a single SRE-binding site was sufficient for Lana-1 to stimulate transcription, demonstrated by activation of the wild-type c-fos transcription factor promoter (Fig. 3). Furthermore, we found that Lana-1 specifically interacted with the transcription factor SRF, which binds to and activates promoters containing the SRE (Fig. 4). Expression of c-fos affects the regulation of many genes involved in cell growth, differentiation and transformation, and its activation is strictly regulated (Angel & Karin, 1991; Kovary & Bravo, 1991). As Lana-1 is one of the few proteins expressed during latency in infected spindle cells, the main constituent of KSHV tumours, its capacity to activate SRE-containing promoters may play a major role in the pathogenesis of KSHV infection, for example by upregulating cytokines such as IL-6 and growth factors such as
vascular endothelial growth factor that have been found to be significantly enhanced upon KSHV infection and that are known to be regulated by MAPKs. In addition, recent studies have shown an interaction between the human T-cell leukemia virus type 1 Tax protein and SRF, which enhances binding to SRE and is suggested to contribute to

Fig. 6. Lana-1 interacts with the Mediator complex. (a) Lana-1 interacts with the endogenously expressed Mediator complex protein MED15. Lysates of HEK293 cells transiently transfected with Lana-1 were precipitated with anti-Lana-1 or anti-MED15 monoclonal antibody (mAb). The blot was reacted with either of the two antibodies or an antibody against the MED7 Mediator complex protein. (b) Lana-1 interacts with the Mediator complex protein MED25. Lysates of HEK293 cells transiently transfected with Lana-1 were precipitated with anti-Lana-1 or anti-MED25 mAb, and binding proteins were detected by Western blot (WB) analysis with anti-Lana-1, anti-MED25 or anti-MED7 mAb. Lanes 1–4, endogenously expressed MED25; lanes 5–8, overexpressed MED25. (c) Lana-1 interacts with the Mediator complex protein MED23. HEK293 cells were transiently transfected with Lana-1 and precipitated with anti-Lana-1 or anti-MED23 mAb. Western blot analysis with anti-Lana-1 or anti-MED23 mAb detected Lana-1 or endogenously expressed MED23. (d) Lana-1-mediated activation of SRE was eliminated by the NTD of MED25. HEK293 cells were transfected with pSRE-luc or pGLMRG5 luciferase plasmids and Lana-1, dominant-positive Ras V12, full-length VP16 or the H1 domain of VP16 as indicated, or with pCR3-GFP (negative control).
Fig. 7. The N terminus of Lana-1 binds to the Mediator complex protein MED25. (a) Schematic diagram of the Lana-1 deletion constructs used. The ability to bind to MED25 or activate the SRE is summarized on the right. (b) The C-terminal Lana-1 domain does not interact with MED25 in CoIP experiments. Plasmids encoding the Lana-1 mutants were transfected into HEK293 cells, immunoprecipitated as indicated, separated by SDS-PAGE, blotted and examined by immunostaining. (c) The N-terminal Lana-1 domain interacts with MED25 in GST pull-down assays. The GST-tagged proteins expressed in BL21 bacteria and bound to GST beads were incubated overnight with the cell lysates of HEK293 cells transfected with MED25. The binding reaction was analysed by Western blotting (WB) using an anti-MED25 antibody (top panel). The GST–Lana-1 fusion proteins used in the binding are shown on a Coomassie blue-stained SDS-polyacrylamide gel (asterisks, bottom panel). (d) The C-terminal domain of Lana-1 is required for SRE activation. HEK293 cells were co-transfected with the SRE reporter plasmid and either a negative control (pCR3-GFP), positive control (Ras V12), wild-type Lana-1 or the C-terminal deletion mutants L25 and L26. Activation is presented as fold increase above the background level.
transformation via regulation of virus/host cell transcription (Winter & Marriott, 2007). Thus, Lana-1 may also be a carcinogenic co-factor by causing aberrant c-fos expression via SRE activation, possibly in combination with manipulation and activation of c-myc (Liu et al., 2007).

Physiological activation of the SRE by Ras V12 occurs following a sequential phosphorylation of the Raf–MEK–ERK signalling cascade. Using luciferase reporter assays in conjunction with chemical inhibitors of MEK1/2 phosphorylation, we found that Lana-1 also uses this MAPK pathway to activate the SRE (Fig. 5). These data are consistent with recent findings that KSHV infection results in activation of the phosphatidylinositol 3-kinase–protein kinase C–MAP/ERK kinase signalling cascade (Naranatt et al., 2003; Sharma-Walia et al., 2005). MAPK pathways are implicated in many stages of KSHV infection, including the establishment of primary infection, lytic replication and reactivation from latency (Pan et al., 2006; Xie et al., 2008). As Lana-1 is expressed in both lytically and latently infected KSHV-positive cells, it may be exploited by KSHV to activate SRE and MAPK pathways and regulate replication during each replication phase. Lana-1 also plays a crucial role in the maintenance of latency by binding to and repressing ORF50, the replication and transactivator protein, which is crucial for the initiation of lytic gene expression (Lan et al., 2004). This repression is eliminated by Pim-1/Pim-3 kinase-mediated phosphorylation of Lana-1, whose expression can be induced by inflammatory cytokines under the control of MAPK signalling (Pan et al., 2006). Our results suggest that there is a crucial homeostasis between repression of ORF50 and activation of cellular signalling pathways, which enables Lana-1 to potentially modulate both lytic and latent replication.

Transcription activation via MAPK pathways requires an interaction between the ternary complex factor TCF and the MED23 subunit of the Mediator complex, a multi-subunit complex of approximately 2 MDa capable of responding to different activators (Casamassimi & Napoli, 2007). The Mediator complex plays a key role in bridging DNA-bound activators of chromatin remodelling with the general transcriptional machinery, particularly RNA polymerase II. Virus modulation of host-cell transcription via interactions with the Mediator complex has been well described: adenovirus large E1A protein interacts with the MED23 subunit via its E1A–CR3 zinc finger (Wang & Berk, 2002); ORFA protein of walleye dermal sarcoma virus co-immunoprecipitates with the Cdk8 and cyclin C subunits (Rovnak & Quackenbush, 2002); herpes simplex virus type 1 VP16 interacts with MED25 via its activation domain (Mittler et al., 2003); and most recently, the transactivation domain of IE62 from VZV initiates transcription via an interaction with MED25 (Yang et al., 2008). In accordance with these studies, we found by CoIP experiments that Lana-1 interacted with several subunits of the Mediator complex – MED15, MED23 and MED25 (Fig. 6). More specifically, the N-terminal domain of Lana-1 interacted with the MED25 subunit of the Mediator complex (Fig. 7). Activation of the SRE by Lana-1, but not by the physiological stimulus Ras V12, was inhibited by the dominant-negative N-terminal domain of MED25. This suggests that Lana-1 uses Mediator complex subunits distinct from those used by Ras V12, in a manner similar to that observed with HSV-1 VP16, and that this subunit of the Mediator complex is essential for transactivation (Fig. 5) (Mittler et al., 2003). In addition, we found that activation of the SRE is dependent on the C terminus of Lana, in a manner similar to that observed for Lana-1-mediated activation of the EBV Cp promoter (Fig. 7d) (Viejo-Borbolla et al., 2003).

In summary, our experiments lead to a model in which Lana-1 first binds to specific transcription factors and subsequently facilitates the attachment of the basal transcriptional machinery through interaction with specific Mediator complex subunits in a manner similar to that suggested for VZV IE62 (Yang et al., 2008) (Fig. 8). Currently, we do not know the stereochemistry of this interaction or whether additional cellular factors are involved. Further analyses must be carried out to clarify the role and function of Lana-1 in this process, as well as to determine whether Lana-1 recruits the Mediator complex to replication compartments, as observed with VZV IE62. These analyses will provide insight not only into a crucial

**Fig. 8.** Lana-1 acts as an adaptor between specific transcription factors and the Mediator complex. Model of the proposed mode of action of Lana-1. The Mediator complex binds to RNA polymerase II and is thought to transduce signals between the transcription machinery and transcription regulatory proteins. We have shown that Lana-1 interacts with the transcription factor SRF and the MED25 subunit of the Mediator complex. We thus hypothesize that Lana-1 contributes to KSHV-induced transcriptional reprogramming by acting as an adaptor between SRF and the Mediator complex.
step of Kaposi’s sarcoma oncogenesis, but also into how transcriptional processes work in non-transformed cells.

**METHODS**

**Plasmids.** The KSHV expression array was generated by recombinatorial cloning (Gateway; Invitrogen) using pDONR207 as an entry vector and the pCR3 (Invitrogen) eukaryotic expression plasmid, modified by inserting the Invitrogen Gateway cassette at the EcoRV site within the multiple cloning site, as a destination vector. Similarly, the plasmid constructs encoding STAT-1, Elk-1 and Spib were generated by recombinatorial cloning of nested PCR products containing attB recombination sites. The first round of PCR used the following transcription factor-specific internal primers: STAT-1 fwd: 5′-AA-AAGCAGGTCCGCCGATgtcaggtgcagac-3′, and STAT-1 rev: 5′-AAGAACGCTGGTTTaatctacaggcaagac-3′; Elk-1 fwd: 5′-AAAA-AGCAGGTCCGCCGATgtgaccattgtgagag-3′, and ELK-1 rev: 5′-AGA-AAGCCTGGTCTGCGTCCTGCGCCTG-3′; and Spib fwd: 5′-AAAAATGACGGTCGCCGATctgtcgctcggtgc-3′ and Spib rev: 5′-AGAAGACTGGTTTcagtgcccaagacagac-3′ (upper-case letters represent regions homologous to the vector transcript, whilst lower-case letters are gene specific). Primary reaction conditions consisted of an initial denaturation step of 94 °C for 2 min 30s followed by 10–15 cycles of 94 °C for 15 s, 55 °C for 60 s and 72 °C for 30 s. The secondary reaction used the same pair of external primers for each transcription factor – attB1 external forward: 5′-GGGGGA-CAAGTTTGTTGAAAAGCAAGC-3′ and attB2 external reverse: 5′-GGGACACATTGTGACAAAGACTGGTTGT-3′ — and the amplification consisted of five cycles of 94 °C for 15 s, 45 °C for 30 s and 68 °C for 15–180 s (60 s per 1 kb). The secondary reaction used the same pair of external primers for each transcription factor – attB1 external forward: 5′-GGGGA-CAAGTTTGTTGAAAAGCAAGC-3′ and attB2 external reverse: 5′-GGGACACATTGTGACAAAGACTGGTTGT-3′ — and the amplification consisted of five cycles of 94 °C for 15 s, 45 °C for 30 s and 68 °C for 15–180 s (60 s per 1 kb). PCR products were run on a 1 % agarose gel, purified by agarose gel electrophoresis and cloned into the pDONR207 plasmid by BP recombination (Gateway; Invitrogen). Subsequently, they were subcloned into the destination vectors pDEST-GKT7 and pDEST-GAD7 by LR recombination (Gateway; Invitrogen). The Lana-1 C-terminal deletion mutants L25 (aa 1–1006) and L26 (aa 1–1055) were created by PCR cloning and mutagenesis from BCP-1 DNA as described previously (Viejo-Borbolla et al., 2003). The plasmids SRE/Luc, mSRE/Luc and mTCP/Luc contained one copy of the wild-type or mutated c-fos SRE-binding site (kindly provided by Dr Jessica Schwartz, University of Michigan) (Liao et al., 1997). The Ras V12 plasmid pRK5c-rycRasV12, used as a positive control for SRE activation, was kindly provided by Dr Alan Hall (Sloan-Kettering Institute, New York, USA). The HIV Tat plasmid pSV2tat72, used as a positive control for HIV LTR activation, was kindly provided through the NIH AIDS Research and Reference Reagent Program (MD, USA) from Dr Alan Frankel (Subramani et al., 1981). The luciferase reporter constructs pAP-1-luc, pNF-xb-luc and pSRE-luc contained 5′–AP-1 (TGACCTAA), 5′–NF-xB (TGGGAGATCTCCCG) and 5′–SRE (AGAGATCCATATTAGGACCT) enhancer elements, respectively (PathDetect; Stratagene). The HIV LTR–luciferase reporter construct was kindly provided by Michael Meisterernst (Helmholtz Zentrum, Munich). The IL-6–luciferase reporter construct was made by Gergana Iotzova (Max-von-Pettenkofer Institute, Munich, Germany). The IL-6–luciferase reporter construct was made by Gergana Iotzova (Max-von-Pettenkofer Institute, Munich, Germany).

**Luciferase assays.** The experiments were carried out either in HEK293 or HaCaT (human endothelial) cells. These cell lines were grown in Dulbecco’s modified Eagle’s medium ( Gibco Invitrogen) containing 4.5 g glucose l−1, 10 mM HEPES, 110 µg sodium pyruvate ml−1, 100 IU penicillin ml−1, 100 µg streptomycin ml−1 and 10 % fetal bovine serum (FBS) in a humidified incubator, with 5 % CO2 at 37 °C. Individual control plasmids (Ras V12 or HIV Tat) or KSHV ORFs and one of the reporter plasmids were transiently transfected by lipofection (HEK293 cells using Effectene, Qiagen; HaCaT cells using LTx, Invitrogen) at a reporter: ORF ratio of 1:5 with a total amount of DNA per sample of 150 ng (48-well plate) or 300 ng (12-well plate). Total DNA was equalized among wells using an empty expression vector. The medium was replaced with 1 % FBS at 24 h post-transfection, and cells were serum-starved for a further 24 h. When required, cells were stimulated with 20 ng ml−1 each of 12-O-tetradecanoylphorbol-13-acetate (TPA) and ionomycin. After a further 24 h, cells were harvested, lysed in 50 µl lysis buffer and substrate (BioAssay Systems; Fig. 1a) or 100 µl l lysis buffer, and luciferase activity was measured by using a commercial luciferase assay system (Promega) in a plate-reading luminometer or a tube luminometer (Berthold). The transfection efficiency was normalized by co-transfection of the pRL-TK plasmid containing Renilla luciferase (Promega) or protein concentration measurements. For promoter stimulation, data represent the mean±SD of three independent experiments performed in triplicate. For inhibitor assays, cells were co-transfected with pSRE-luciferase reporter plasmid and either an empty pCR3 vector or Lana-1 or Ras V12. Cells were treated with 10–20 µM MEK1/2 inhibitors U0126 and PD98059 and harvested the next day and assessed for luciferase activity.

**CoIP.** The following antibodies were used for CoIP: rat monoclonal anti-Lana-1 (ABI), rat monoclonal anti-MED25 clone 9C2, rat monoclonal anti-MEDI5 clone 6C9, rat monoclonal anti-MED7 and anti-MEDI3 (BD Pharmingen), rabbit polyclonal anti-SRF (C termisusus) and mouse monoclonal anti-Myc (Santa Cruz), and rat monoclonal anti-HA (Roche). HEK293 cells were cultured on 10 cm dishes and transfected with 10 µg of each of the two expression plasmids by calcium phosphate transfection. After 24 h, the culture medium was exchanged for fresh medium containing 1 % FCS and the cells were starved for another 24 h. After 48 h, cells were harvested and lysed by incubation for 30 min in 1 ml NP-40 lysis buffer (1 % NP-40, 140 mM NaCl, 5 mM MgCl2, 20 mM Tris/His (pH 7.6), 1 mM PMSF) on ice. Lysates were centrifuged for 15 min at 20 000 g at 4 °C to remove unsolubilized material and pre-cleared with 25 µl protein G–Sepharose (Pharmacia) for 1 h at 4 °C. Subsequently, antibodies and protein G–Sepharose were added and incubated with the lysates for 12 h. After resuspension in 2 × sample buffer (100 mM Tris/His (pH 6.8), 200 mM dithiothreitol, 4 % SDS, 0.2 % bromophenol blue, 20 % glycerol), samples were boiled for 5 min, separated by 7.5–10 % SDS-PAGE and transferred to nitrocellulose membranes (Schleicher & Schuell). Protein bands were visualized with peroxidase-conjugated secondary antibodies and enhanced chemiluminescence (Pharmacia). CoIP between Lana-1 (cloned in pDEST-GAD7) and the transcription factors Elk-1, STAT-1 and Spib (cloned in pDEST-GKT7) were performed using total cell lysates of HEK293 cells infected with recombinant vaccinia virus expressing the T7 RNA polymerase and subsequently transfected. HEK293 cells were cultured on 10 cm dishes and infected with viral T7 RNA polymerase at an m.o.i. of 10 in serum-free medium. The cells were incubated at 37 °C for 1.5 h. Immediately after infection, cells were transfected with 10 µg of each of the two expression plasmids by calcium phosphate transfection as described above and harvested at 24 h post-transfection.

**GST pull-down experiments.** GST-tagged Lana-1 constructs pGL9, pGL12 and pGL13 were generated as described previously, and were generously provided by Dr Diane Hayward (Johns Hopkins School of Medicine, Baltimore, MD, USA) (Krithivas et al., 2000). Overnight cultures of BL21 cells transformed with constructs expressing GST fusion proteins were inoculated into 9 ml fresh Luria broth containing ampicillin (100 µg ml−1) and incubated for 1 h at 37 °C. IPTG was then added to a final concentration of 1 mM and the cells were incubated for an additional 3–4 h at 30 °C. The bacteria were harvested by centrifugation at 2400 g and 4 °C for 15 min. The pellet was dissolved in 500 µl PBS containing 1 mM PMSF (Sigma) and...
4 mM Pefablock (Roche) and subsequently sonicated three times for 5 s each (Branson 450 Sonifier). Triton X-100 was added to a final concentration of 1 %, and the solutions were mixed and centrifuged at 4 °C for 10 min at 16 400 g. The cleared supernatants were collected and mixed with 50 μl washed glutathione beads. The protein lysates were incubated with the glutathione beads for 30 min at room temperature. The beads were centrifuged and washed three times with washing buffer [50 mM Tris/HCl (pH 7.8), 150 mM NaCl, 0.5 mM EDTA, 0.1 % NP-40]. Subsequently, the beads were centrifuged, washed three times and boiled for 10 min at 95 °C in 2 × sample buffer (as above).

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