Kaposi’s sarcoma-associated herpesvirus (KSHV) is a latent gammaherpesvirus that typically infects B-lymphocytes in the human body, and its reactivation can result in the development of a variety of diseases, including Kaposi’s sarcoma, primary effusion lymphoma, and multicentric Castleman’s disease (Jordan et al., 1991). The exact mechanism for the reactivation of KSHV latency is still elusive. Generally, a lytic cycle of KSHV infection can be induced in cells harbouring latent KSHV by treating with phorbol ester (TPA). However, the exact signalling events that lead to the reactivation of KSHV lytic infection are still elusive. Here, we report on the role of MEK/ERK signalling in the reactivation of KSHV latent infection by treatment with TPA. We investigated whether TPA could induce a lytic cycle of KSHV infection in a primary effusion lymphoma (PEL)-derived cell line, BCBL-1, which harbours latent KSHV infection. To do this, we attempted to decipher the signalling events that are critical for the reactivation of KSHV latency in a primary effusion lymphoma (PEL)-derived cell line.

The MAPK pathway is one of the better-studied signal-transduction pathways. TPA is reported to induce protein kinase C (PKC) and Raf/MEK/ERK signalling, along with a variety of other signalling pathways (Gao et al., 2001; Jang et al., 2005). In the present study, we tested whether TPA could induce ERK1/2 activity in KSHV-infected BCBL-1 cells. We observed a sustained three- to fourfold enhancement in ERK1/2 activity due to TPA treatment. This increase in TPA-induced ERK1/2 activity was largely nullified by treating cells with 10 μM of the MEK inhibitor U0126 (Biosource) (Fig. 1a), but not by DMSO, the vehicle for U0126 (data not shown). U0126 treatment of uninduced cells also lowered ERK1/2 activity significantly (Fig. 1a). The results indicate clearly that TPA can also induce ERK1/2 activity in BCBL-1 cells. To investigate a possible role for MEK/ERK signalling in TPA-induced KSHV lytic cycle infection, we monitored expression of ORF73 (LANA), ORF8 (gB) and β-actin genes by RT-PCR in uninduced and TPA-induced BCBL-1 cells. Expression of KSHV ORF8 (a lytic-cycle gene) was significantly higher in cells treated with TPA than in uninduced cells (Fig. 1b). There was also a slight increase in the expression of ORF73 in TPA-induced BCBL-1 cells compared with uninduced cells (Fig. 1b). Interestingly, U0126 treatment of cells significantly nullified the effects of TPA on the enhanced expression of ORF73 and ORF8 (Fig. 1b). No significant change in TPA-enhanced expression of ORF73 and ORF8 was observed in cells treated with DMSO (data not shown). Further, significant differences in the levels of β-actin were not detected between respective treatments (data not shown), demonstrating the specificity of the effect of TPA on the expression of KSHV-encoded genes. Similar results were observed when PD98059 was used to inhibit MEK activity (data not shown).

B-Raf is believed to be the main regulator of MEK/ERK activity (O’Neill & Kolch, 2004). It was demonstrated recently that, unlike A-Raf and Raf-1, B-Raf depletion by small interfering RNA (siRNA) inhibits ERK1/2 activity (Karasarides et al., 2004). In a recently concluded study, we demonstrated the ability of B-Raf siRNA to significantly and specifically lower B-Raf/MEK/ERK signalling in BCBL-1 cells (Akula et al., 2005). RT-PCR data demonstrated that the transfection of B-Raf siRNA into BCBL-1 cells significantly nullified the effects of TPA on the enhanced expression of ORF73 and ORF8 (Fig. 1c). In contrast, no significant
change in TPA-enhanced expression of ORF73 and ORF8 was observed in cells transfected with non-specific siRNA [(NS)siRNA] (Fig. 1c). Significant differences in the levels of β-actin were not detected between respective treatments (data not shown). We confirmed the results from semi-quantitative RT-PCR by analysing the expression of KSHV-encoded late-lytic protein (gB) by a more reliable and quantitative approach using flow cytometry, as per earlier protocols (Akula et al., 2002). BCBL-1 cells stimulated with TPA (Fig. 1e) expressed gB protein at higher levels than in the uninduced cells (Fig. 1d). Transfection of TPA-induced BCBL-1 cells with B-Raf siRNA lowered the surface expression of gB significantly (Fig. 1f). Transfection of TPA-induced BCBL-1 cells with (NS)siRNA did not alter the surface expression of gB significantly, suggesting the specificity of B-Raf siRNA in lowering gB expression (Fig. 1g). We simultaneously monitored expression of gB on target cells by confocal microscopy (Fig. 2a). This was primarily done to rule out any non-specific interactions by antibodies to gB (Akula et al., 2001). The results from studies involving the use of confocal microscopy corroborate those obtained by using flow cytometry. Antibodies to gB specifically reacted with 17–25 % of BCBL-1 cells that were treated with TPA, compared with 1–3 % in the case of untreated cells (Fig. 2a). Rabbit preimmune IgG did not react with the target cells (data not shown). Our results support earlier findings that TPA treatment induces expression of KSHV-encoded late proteins in only 20–25 % of cells (Renne et al., 1996).

If B-Raf siRNA could lower the expression of KSHV late protein (gB) in TPA-induced BCBL-1 cells, we hypothesized that the transfection of cells with B-Raf siRNA would lower the number of infectious KSHV particles produced by the target cells. Supernatants from TPA-induced BCBL-1 cells contained a significantly greater number of infectious KSHV particles than supernatants derived from uninduced cells (Fig. 2b). Transfection of cells with B-Raf siRNA significantly lowered the number of infectious KSHV particles.
particles in the supernatants obtained from TPA-induced cells compared with transfection of cells with (NS)siRNA. In our experiments using flow cytometry, confocal microscopy and infection assays, transfection of cells with B-Raf siRNA could not lower either the expression of lytic-cycle KSHV proteins or the number of infectious particles produced to zero levels, suggesting that B-Raf/MEK/ERK signalling is probably one of several mechanisms involved in reactivation of KSHV infection of cells. ORF73 is considered to play a major role in regulating viral latency, persistence and transformation (An et al., 2005; Friborg et al., 1999; Fujimuro et al., 2005; Radkov et al., 2000). The ORF73 gene product not only regulates KSHV latency, but also viral lytic replication (Lan et al., 2004). This is probably one of the reasons for the marginal increase in ORF73 expression upon induction of lytic-cycle replication (Jenner et al., 2001).

Finally, we analysed the effect of transient transfection of BCBL-1 cells with a vector encoding MEK1 (pCMV-MEK1; Clontech) on inducing the lytic cycle of KSHV infection. BCBL-1 cells were uninduced, TPA-induced or transfected with B-Raf or (NS)siRNA and then induced with TPA. Supernatants were collected at 72 h after TPA induction and tested for KSHV infection on HFF cell monolayers. HFF cells grown to 75% confluence in a one-well flaskette glass slide (10 cm²; Nunc) were infected with the above supernatant for 8 h at 37°C. The cells were washed twice with Dulbecco’s modified Eagle’s medium and further incubated with growth medium at 37°C. After 3 days, KSHV infection was monitored by performing an immunoperoxidase assay to detect ORF73 expression (Hamden et al., 2004). The total number of KSHV-infected cells counted in four different slides was presented for comparison between different treatments. Mean values (n=3) on the columns with different superscripts are statistically significant (P<0.05) by least significant difference (LSD).

**Fig. 2.** (a) Confocal microscopy to analyse the expression of KSHV gB on target cells. Target cells were fixed in 0.1% paraformaldehyde at +4°C for 10 min. The cells were incubated sequentially with rabbit polyclonal antibodies to gB and FITC-anti-rabbit antibodies. Finally, the stained cells were further incubated for 20 min on ice with 5 µM SYTO Red (a nuclear stain; Invitrogen) before being washed in ice-cold PBS. These cells were spotted on glass slides and analysed with a laser-scanning LSM 510 Carl Zeiss confocal microscope. The mean number of positive cells counted over five random fields was used for comparison and analysis. Arrowheads indicate cells that are positive for gB expression. Magnification, ×62. (b) Inhibition of B-Raf-associated signalling lowers the lytic cycle of KSHV infection. BCBL-1 cells were uninduced, TPA-induced or transfected with B-Raf or (NS)siRNA and then induced with TPA. Supernatants were collected at 72 h after TPA induction and tested for KSHV infection on HFF cell monolayers. HFF cells grown to 75% confluence in a one-well flaskette glass slide (10 cm²; Nunc) were infected with the above supernatant for 8 h at 37°C. The cells were washed twice with Dulbecco’s modified Eagle’s medium and further incubated with growth medium at 37°C. After 3 days, KSHV infection was monitored by performing an immunoperoxidase assay to detect ORF73 expression (Hamden et al., 2004). The total number of KSHV-infected cells counted in four different slides was presented for comparison between different treatments. Mean values (n=3) on the columns with different superscripts are statistically significant (P<0.05) by least significant difference (LSD).
marginal increase in the expression of ORF73 in BCBL-1 cells transfected with pCMV-MEK1 compared with either untransfected cells or those transfected with empty vector (Fig. 3b). There was no significant difference in the levels of β-actin detected between respective treatments (data not shown). The RT-PCR data were confirmed by analysing the expression of KSHV gB by flow cytometry. BCBL-1 cells transfected with pCMV-MEK1 expressed elevated levels of gB on the cell surface compared with cells that were either untransfected or transfected with empty vector (Fig. 3c–e). The above results were further confirmed by confocal microscopy, as done previously (Fig. 2a). Antibodies to gB specifically reacted with 13–17 % of BCBL-1 cells that were transfected with pCMV-MEK1, compared with 1–3 % in the case of cells that were untransfected or transfected with empty vector (data not shown).

Additionally, supernatants from BCBL-1 cells transfected with pCMV-MEK1 contained a significantly greater number of infectious KSHV particles than supernatants derived from both untransfected cells and those transfected with empty vector (Fig. 3f). The reactivation observed due to the overexpression of MEK1 was not comparable to that observed in cells treated with TPA; this could be due to the fact that TPA treatment of cells activates several other signalling cascades that may be critical and have a synergistic effect on the reactivation of virus infection. Interestingly, overexpression of A-Raf, B-Raf or Raf-1 isoforms did not
initiate reactivation of KSHV latent infection in human foreskin fibroblasts (HFFs) (Akula et al., 2004). Transfection of HFF cells with pCMV-MEK1 also failed to initiate reactivation of KSHV infection (data not shown). Incidentally, TPA treatment of HFFs does not reactivate KSHV latency (Vieira et al., 2001). Hence, we concluded that this role of B-Raf/MEK/ERK signalling to initiate the lytic cycle of KSHV infection is target cell-specific. We speculate it to be a predominantly B cell-restricted process.

Earlier studies identified TPA to activate PKC signalling (Jang et al., 2005). TPA-induced PKC-δ was concluded to be an essential mediator of KSHV reactivation (Deutsch et al., 2004). However, these authors demonstrated that the stimulation of PKC-δ was not sufficient to induce KSHV lytic reactivation. TPA can also activate ERK via the PKC/Raf/MEK signalling (Jang et al., 2005; Marquardt et al., 1994). Such a diverse role for the Raf/MEK/ERK signalling pathway in mediating lytic infection of EBV has been reported previously (Fenton & Sinclair, 1999; Satoh et al., 1999).

Our results identified B-Raf/MEK/ERK signalling as one of the mediators that is able to reactivate KSHV latency in PEL cells. KSHV-associated pathogenesis is mediated by a complex interplay between inflammatory cytokines (ICs) and growth factors (GFs) (Ensoli et al., 2001). Constitutive activation of the components (Ras/Raf) of the MAPK pathway of signalling has been a common feature associated with KSHV pathogenesis (Faris et al., 1998). Interestingly, B-Raf-associated signalling plays multiple roles in KSHV pathogenesis by regulating expression of a variety of ICs/GFs (Giri et al., 2003; Man et al., 2005; Matsubara et al., 2005; Nakayama et al., 2003), including that of VEGF (Akula et al., 2005). We hypothesize that B-Raf/MEK/ERK signalling triggers KSHV lytic replication by its ability to modulate the expression of ICs/GFs. Such a role for MAPK signalling in the activation of HIV-1 latency has been reported previously (Yang et al., 1999). These authors demonstrated the ability of MAPK to modulate cytokine expression as one of the reasons for reactivation of human immunodeficiency virus type 1 latency. Having said this, the obvious question would be about the manner in which this B-Raf/MEK/ERK signalling regulates the reactivation process in vivo. At this point, based on our recently published study (Bryan et al., 2006), we conjecture a complex, intricate interaction between tightly regulated cell-cycle events and the MAPK pathway to play a crucial role in the actual switch from latent to lytic cycles of KSHV infection. Taken together, the present findings will serve as a starting point in unravelling the mystery surrounding virus latency. Future studies are focused on deciphering the specific signature of cells critical for the B-Raf/MEK/ERK signalling-induced KSHV lytic cycle of infection.

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